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CORTICOTROPIN RELEASING FACTOR RECEPTORS AND AGONISTIC BEHAVIOR IN SYRIAN HAMSTERS

by

ALICIA N. FARUZZI

Under the Direction of Kim L. Huhman, PhD

ABSTRACT

Social conflict is a part of everyday life, and it can be a potent stressor for both humans and other animals. In the laboratory, when two Syrian hamsters (*Mesocricetus auratus*) compete for territory, a dominance hierarchy is quickly formed. Becoming subordinate is a significant stressor resulting in increased release of adrenocorticotrophic hormone, β -endorphin, and cortisol. Defeated hamsters will also subsequently fail to display territorial aggression in future social encounters and will instead display increased submissive behavior, even in the presence of a smaller, non-aggressive intruder. This change in behavior is consistent and long-lasting and has been termed conditioned defeat (CD).

Corticotropin releasing factor (CRF) is an important neuropeptide in the control of the hypothalamo-pituitary-adrenal (HPA) axis response to stress. It is also involved in a number of behaviors such as anxiety, stress responding, food intake, learning, and memory. The widespread distribution of CRF, CRF-like peptides, and CRF receptors, particularly in brain sites related to anxiety, fear, and stress responses, suggests a role for CRF and CRF-like peptides in modulating emotional responses other than via HPA axis activity.

It has also been shown that CRF may have a role in the acquisition and expression of CD. Non-specific and CRF type 2-specific CRF antagonists reduce the acquisition and expression of CD in male hamsters while injection of a CRF type 1-specific antagonist does

not. Therefore, the goal of this dissertation was to investigate the role of CRF type 1 and 2 receptors in CD in hamsters and to identify neuroanatomical locations where CRF may be acting. It was found that non-specific or CRF type 1 receptor specific agonists enhance the expression, but not acquisition, of CD. Further, these agonists appear to enhance aggressive behavior in animals that were not previously defeated, suggesting a modulatory role for CRF type 1 receptors in agonistic behavior that depends on an animal's previous social experience. Further, localization of CRF receptors was determined in hamster brain in sites thought important for CD and agonistic behavior, but changes in receptor binding following defeat were not observed. Implications of these results and future directions are discussed.

INDEX WORDS: aggressive behavior, submissive behavior, stress, conditioned defeat, autoradiography, bed nucleus of the stria terminalis, anxiety, ovine CRF

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IN SYRIAN HAMSTERS

by

ALICIA N. FARUZZI

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2005

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Alicia N. Faruzzi
2005

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ALICIA N. FARUZZI

Major Professor:	Kim L. Huhman
Committee:	H. Elliott Albers
	Aras Petrulis
	Larry J. Young

Electronic Version Approved:

Office of Graduate Studies
College of Arts and Sciences
Georgia State University
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DEDICATION

For Mom, with love and gratitude

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There have been so many people who have helped me get to this point, many of whom have moved on to greener pastures before me. To them I say thank you and good luck. To my mentor and friend, Kim, thank you for all you have taught me, all the support you have given me, and all the random conversations we had we had in your office about absolutely nothing scientifically-related whatsoever. To Elliott I owe much gratitude for helping teach me how to be a scientist as well as how to do a proper tequila shot. To Aras and Larry, thank you for teaching me and guiding me through my dissertation. Lauren, Jeris, Alisa, Matt, and Chris, no matter whether I'm a bringer or a buyer, lunch will never be the same without you- though I would probably still be at GSU without your help and encouragement over the years! Matia, Desiree, Stacie, and Michelle, what can I say, except, "See you in Vegas!" I love you all. Dad, every time you try and fail to say what I do for a living, you make me feel so smart, and then I blush- thank you. Mom, I don't know how to express my gratitude to you; gratitude isn't even the right word. I think the best I can do is continue to say to you the same two things that I've been saying since I turned 18: you were right, and thank you.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	v
LIST OF TABLES.....	viii
LIST OF FIGURES.....	ix
LIST OF ABBREVIATIONS.....	x
CHAPTER	
1 INTRODUCTION.....	1
Stress.....	1
Conditioned and unconditioned fear.....	2
The neurobiology of agonistic behavior.....	3
Corticotropin releasing factor and its receptors.....	5
Corticotropin releasing factor and stress.....	8
Corticotropin releasing factor as a modulator of fear and anxiety...	10
Syrian hamsters and conditioned defeat.....	11
The neurobiology of conditioned defeat.....	13
Scope of this dissertation.....	13
2 CENTRAL CORTICOTROPIN RELEASING FACTOR MODULATES THE EXPRESSION OF AGONISTIC BEHAVIOR IN SYRIAN HAMSTERS.....	15
Introduction.....	18
Methods.....	21
Results.....	26
Discussion.....	28

3	PREVIOUS SOCIAL EXPERIENCE MODULATES THE EFFECTS OF CORTICOTROPIN RELEASING FACTOR ON AGONISTIC BEHAVIOR IN SYRIAN HAMSTERS.....	36
	Introduction.....	39
	Methods.....	42
	Results.....	48
	Discussion.....	50
4	CORTICOTROPIN RELEASING FACTOR TYPE 1 AND 2 RECEPTOR DISTRIBUTION IN SYRIAN HAMSTER BRAIN.....	56
	Introduction.....	58
	Methods.....	61
	Results.....	65
	Discussion.....	68
5	CORTICOTROPIN RELEASING FACTOR RECEPTOR BINDING IN SYRIAN HAMSTER BRAIN IS NOT ALTERED BY ACUTE AGONISTIC INTERACTION.....	81
	Introduction.....	83
	Methods.....	85
	Results.....	89
	Discussion.....	89
6	GENERAL DISCUSSION.....	93
	Synthesis of this dissertation.....	93
	Future directions.....	97
	REFERENCES.....	101
	APPENDIX.....	115

LIST OF TABLES

TABLE 4.1	Abbreviations.....	79
TABLE 4.2	^{125}I -sauvagine binding to CRFR1 and CRFR2 in Syrian hamster brain.....	80

LIST OF FIGURES

FIGURE 2.1	Means \pm SEM of cortisol in male hamsters receiving metyrapone (0.0, 50.0, 100 mg/kg SC).....	33
FIGURE 2.2	Means \pm SEM of duration of submissive, aggressive, social and nonsocial behavior in male hamsters receiving metyrapone either a) before training (acquisition effects) or b) before CD testing (expression effects).....	34
FIGURE 2.3	Means \pm SEM of duration of submissive, aggressive, social and nonsocial behavior in defeated and non-defeated male hamsters receiving r/hCRF either a) before training (acquisition effects) or b) before CD testing (expression effects).....	35
FIGURE 3.1	Means \pm SEM of duration of submissive, aggressive, social and nonsocial behavior in defeated and non-defeated male hamsters receiving oCRF or vehicle before CD testing.....	54
FIGURE 3.2	Means \pm SEM of duration of submissive, aggressive, social and nonsocial behavior in a) non-defeated and b) defeated male hamsters receiving ASVG/vehicle and oCRF/vehicle before CD testing.....	55
FIGURE 4.1	CRFR total binding (labeled with “a”) and CRFR2 binding (labeled with “b”) throughout hamster brain from prefrontal cortex to cerebellum (numbered 1-38).....	73
FIGURE 4.2	Adjacent thionin-stained slides to autoradiography slides.....	76
FIGURE 4.3	Adjacent AChE-stained slides of the extent of the amygdala.....	78
FIGURE A.1	Represents potential circuitry involved in the control of agonistic behavior by CRFR1 in the LS.....	116
FIGURE A.2	Represents potential circuitry involved in the control of agonistic behavior by CRFR1 in the BST.....	117

LIST OF ABBREVIATIONS

AChE	acetylcholinesterase
ACTH	adrenocorticotrophic hormone
AH	anterior hypothalamus
ANOVA	analysis of variance
Arc	arcuate nucleus
AStr	amygdalostriatal transition area
ASVG	antisauvagine-30
BLA	basolateral nucleus of the amygdala
BLA	basolateral amygdala (anterior/posterior)
BLV	ventral basolateral amygdala
BMA	anterior basomedial amygdala
BMP	posterior basomedial amygdala
BST	bed nucleus of the stria terminalis
BSTA	anterior bed nucleus of the stria terminalis
BSTL	lateral bed nucleus of the stria terminalis
BSTPI	posterointermediate bed nucleus of the stria terminalis
BSTPL	posterolateral bed nucleus of the stria terminalis
BSTPM	posteromedial bed nucleus of the stria terminalis
CD	conditioned defeat
Ce	central nucleus of the amygdala
Ce	central amygdala
Cg	cingulate cortex
CG	central gray

Cg1	cingulate cortex 1
Cg2	cingulate cortex 2
CORT	cortisol
CPu	caudate putamen
CRF	corticotropin releasing factor
CRFR1	corticotropin releasing factor type 1 receptor
CRFR2	corticotropin releasing factor type 2 receptor
DG	dentate gyrus
DMSO	dimethyl sulfoxide
DR	dorsal raphe
DW	defensive withdrawal
GABA	gamma-aminobutyric acid
H ₂ O	water
HPA	hypothalamo-pituitary-adrenal
I	intercalated amygdala
ICV	intracerebroventricular
IL	infralimbic cortex
La	lateral amygdala (anterior/posterior)
LC	locus coeruleus
LH	lateral hypothalamus
LS	lateral septum
LSD	least significant difference
LV	lateral ventricle
Me	medial amygdala

MeA	anterior medial amygdala
MeP	posterior medial amygdala
MgCl	magnesium chloride
MHZ	medial hypothalamic defense system
MnR	median raphe
mRNA	messenger RNA
NMDA	N-methyl-D-aspartate
NS	non-specific binding
oCRF	ovine corticotropin releasing factor
Pa	paraventricular nucleus of the hypothalamus
PAG	periaqueductal gray
PBS	phosphate buffered saline
PLCo	posterior lateral cortical amygdala
PMCo	posterior medial cortical amygdala
PMnR	paramedian raphe
PrL	prelimbic cortex
r/hCRF	rat/human corticotropin releasing factor
R1	corticotropin releasing factor type 1 receptor-specific binding
R1R2	total corticotropin releasing factor receptor binding
R2	corticotropin releasing factor type 2 receptor-specific binding
RIA	radioimmunoassay
S	subiculum
SC	subcutaneous
SCN	suprachiasmatic nucleus of the hypothalamus

SEM	standard error of the mean
Sol	nucleus of the solitary tract
SuG	superficial gray layer of the superior colliculus
UCN	urocortin
UCNII	urocortin II (or human urocortin II)
UCNIII	urocortin III
VLH	ventrolateral nucleus of the hypothalamus
VMH	ventromedial nucleus of the hypothalamus
VMHL	lateral ventromedial nucleus of the hypothalamus
VMHM	medial ventromedial nucleus of the hypothalamus

INTRODUCTION

This dissertation examines the role of corticotropin releasing factor receptors in agonistic behavior and in conditioned defeat in Syrian hamsters. Data from a number of research areas including stress, anxiety, conditioned and unconditioned fear, aggression, and defense are relevant to this topic. These areas are reviewed briefly in the following sections. Background material necessary for an understanding of corticotropin releasing factor and its receptors, both on a molecular level and as a modulator of behavior, are also presented.

Stress

Stress is the nonspecific response of the body to any demand, particularly one that is noxious (Selye, 1998). The body has a given set of responses that it uses to deal with various stressors, and prolonged or severe stress can cause illness (Selye, 1979). Stressful life events are linked to an increased chance of developing several psychological disorders, including affective or anxiety disorders (Holsboer, 1988; Barden, 1996; Plotsky *et al.*, 1998; Mitchell, 1998; Arborelius *et al.*, 1999).

Numerous animal models have been utilized to study stress, such as restraint, foot shock and forced swim. However, many of these models use physical, sometimes painful, stimuli to evoke stress. Although these studies have provided important insights into the physiological basis of stress, they may not be considered ethologically valid models of psychological stress. For example, foot shock is not a stressor that an animal is likely to encounter in the wild, thus it may elicit a stress response that is much stronger than would be elicited in response to a more naturalistic stressor. In contrast, a more relevant stressor, such as social conflict or predation, which an animal might encounter in the wild, may elicit a more natural stress response. Some

current, more ethologically valid models of stress include features like maternal deprivation (Arborelius *et al.*, 1999; Lehmann *et al.*, 1999; Hsu *et al.*, 2003; Romeo *et al.*, 2003; Vazquez *et al.*, 2003) or social interaction or conflict (Korte *et al.*, 1990; Haller *et al.*, 1996; Blanchard *et al.*, 2001; Cacho *et al.*, 2003; Summers *et al.*, 2003).

Social conflict is necessary for the development of social relationships in many species, both human and non-human. When conflict arises, the outcome (i.e., whether the individual “wins” or “loses”) may have a strong and lasting impact. Animals can encounter social conflict in such contexts as competition for food or access to females or territories. Results of being defeated during an agonistic encounter can include increased heart rate, elevated blood pressure, and suppressed immune function (Bohus *et al.*, 1993; Blanchard *et al.*, 1995; Meehan *et al.*, 1995), as well as activation of the HPA axis and behavioral changes like decreased reproductive, exploratory, or aggressive behavior, decreased food and water intake, and increased anxiety (Heinrichs *et al.*, 1992; Potegal *et al.*, 1993; Rodgers and Cole, 1993; Albonetti and Farabollini, 1994).

Conditioned and Unconditioned Fear

There has been extensive research investigating the neuroanatomy of fear, the control of which appears to lie in the amygdala or BST. Several subnuclei of the amygdala appear to be critical components of the circuitry in the brain responsible for the acquisition and the expression of conditioned fear (Davis, 1997). The BLA receives information from cortical areas involved in processing sensory input. The BLA projects to the Ce which then projects to sites in the hypothalamus and brain stem that are responsible for mediating physiological and behavioral responses to stressors. Some of these nuclei and their corresponding functions include the lateral hypothalamus (activation of the sympathetic nervous system),

the paraventricular nucleus (glucocorticoid release), the ventral tegmental area, locus coeruleus and basal forebrain (increased attention and vigilance), and the PAG (freezing or fleeing). The amygdala also shares reciprocal projections with the BST, forming what is sometimes referred to as the extended amygdala (McDonald, 2003).

Whereas the Ce is responsible for controlling conditioned fear responses via its projections to the hypothalamus and brainstem, the BST, or more specifically the lateral BST (BSTL), appears to be more involved in controlling the various physiological and behavioral responses to unconditioned fearful stimuli (Walker and Davis, 1997; Davis, 1997; Lang *et al.*, 2000). The BSTL receives input from the BLA and has similar outputs as the Ce (Davis and Shi, 1999). Lesions of the BST block unconditioned fear responses in models such as light- or CRF-enhanced startle in rats but do not block fear-potentiated startle, a measure of conditioned fear. Conversely, lesions of the Ce block fear-potentiated startle but not light- or CRF-enhanced startle. Finally, inactivation of the BLA attenuates both types of fear responses. These findings have supported the hypothesis that there are two central fear circuits, one for conditioned fear that is mediated by the Ce and one for unconditioned fear that is mediated by the BSTL (Walker and Davis, 1997; Davis and Shi, 1999).

The Neurobiology of Agonistic Behavior

Agonistic behavior refers to the profile of behaviors exhibited during social conflict. It includes aggressive behaviors, such as threat, attack, and pursuit, as well as defensive and submissive behaviors such as defensive postures and flight (Albers *et al.*, 2002). Social communication is another aspect of agonistic behavior, but the neurobiology of communicative behavior will not be discussed here.

There are similarities and differences in the neural correlates controlling aggressive and defensive behavior. The hypothalamus appears to lie at the center of either system (Delville *et al.*, 2000; Canteras, 2002). In the control of offensive aggression, the AH receives olfactory and vomeronasal inputs from the amygdala (Me) and BST, as well as somatosensory inputs from the PAG and autonomic inputs from the parabrachial nucleus (Delville *et al.*, 2000). These sites, as well as the LS, share reciprocal connections with the AH, and some of them show Fos activation following bouts of offensive aggression (particularly Me, BST, and PAG, and, of course, the AH). The VLH also shows Fos activation and shares reciprocal connections with the AH, and stimulation of the VLH elicits offensive aggressive behaviors (Delville *et al.*, 2000).

The AH and the VMH are also important components of what Canteras (2002) refers to as the medial hypothalamic defense system, or MHZ. Further, many of the same inputs important for aggressive behavior are involved in the control of defensive behavior, and most of these sites also show neuronal activation following social defeat (Kollack-Walker and Newman, 1995; Kollack-Walker *et al.*, 1997; Martinez *et al.*, 1998; Martinez *et al.*, 2002). For example, the AH and VMHM receive inputs from the amygdala and BST that convey sensory information necessary to trigger innate defensive responses, while the parabrachial nucleus conveys information to the AH and VMHM about noxious stimuli (Canteras, 2002). The role of the LS is likely similar in both offensive and defensive behavior. Specifically, the LS appears to exert inhibitory control over the initiation of aggressive behavior from the AH (Canteras, 2002). While this reciprocal pathway may be more important for receiving feedback during offensive behavior (Delville *et al.*, 2000), it would be important to have a direct pathway for inhibiting aggression when defensive behavior is the appropriate response.

Thus, the LS may act as a “switch,” turning attack on during offensive aggression or turning it off to allow for defense.

Most outputs of the MHZ are to the same regions from where the inputs originate, likely forming feedback loops (with the LS, amygdala, and BST, specifically). However, the VMHM projects to the PAG to organize defensive responses. Additionally, the AH and VMHM both project to the dorsomedial hypothalamus, which coordinates behavioral, autonomic, and endocrine responses to acute stress (Canteras, 2002). At this point, defensive and aggressive control differs greatly, because the stress response is an important element of defensive behavior. Activation is seen in key sites involved in the stress response following acute defeat (Kollack-Walker and Newman, 1995; Kollack-Walker *et al.*, 1997; Martinez *et al.*, 1998; Martinez *et al.*, 2002), making stress circuitry a critical component of the neurocircuitry controlling defensive behavior.

Corticotropin Releasing Factor and its Receptors

CRF is a 41-amino acid peptide (Vale *et al.*, 1983) located in the anterior pituitary and throughout the brain (Chappell *et al.*, 1986; Eckart *et al.*, 1999; Eckart *et al.*, 2002). There are a number of peptides in the CRF family (see below), and they are involved in a range of behaviors such as anxiety, stress responding, food intake, and learning/memory (Eckart *et al.*, 1999; Reyes *et al.*, 2001; Grammatopoulos and Chrousos, 2002). There are currently two known receptors in the CRF family. CRFR1 has one functional splice variant and CRFR2 has three functional splice variants (CRFR2 α , 2 β , 2 γ) (Dautzenberg and Hauger, 2002; Eckart *et al.*, 2002). Both receptors are primarily Gs protein-coupled and they activate adenylyl cyclase to increase cyclic AMP levels, though they are also associated with other signaling pathways (Dautzenberg and Hauger, 2002; Eckart *et al.*, 2002; Grammatopoulos

and Chrousos, 2002). CRFR1 is found throughout the brain in areas important for sensory information processing and motor control, while CRFR2 is more restricted to subcortical regions (Reul and Holsboer, 2002). CRFR1 mRNA has been found in sites including the anterior pituitary, cerebral cortex, cerebellum, amygdala, hippocampal formation, hypothalamus, areas of the brain stem, septum, BST, and olfactory bulb. CRFR2 mRNA has been found in areas including the hippocampal formation, amygdala, LS, BST and areas of the hypothalamus and brainstem (Eckart *et al.*, 1999; Bittencourt and Sawchenko, 2000; Dautzenberg and Hauger, 2002). Binding of agonists to CRFR1 causes HPA axis activation, increased anxiety, increased depression, decreased feeding, and decreased inflammatory response; binding of agonists to CRFR2 causes decreased feeding, decreased gastric emptying, decreased depression (except when administered into the LS), increased vasodilation, and decreased blood pressure (Holsboer, 1999; Steckler and Holsboer, 1999; Arborelius *et al.*, 1999; Eckart *et al.*, 1999; Koob and Heinrichs, 1999; Takahashi, 2001; Dautzenberg and Hauger, 2002; Eckart *et al.*, 2002; Grammatopoulos and Chrousos, 2002). The role that CRFR2 plays in anxiety and stress is not well understood. Some studies and reviews report anxiolytic responses to CRFR2 activation (Eckart *et al.*, 1999; Hashimoto *et al.*, 2001; Dautzenberg and Hauger, 2002; Pellemounter *et al.*, 2002), while other studies report anxiolytic responses with antagonism of CRFR2 (Takahashi *et al.*, 2001) or suggest that CRFR2 activity can produce anxiogenic or anxiolytic properties depending on when testing occurs or where in the brain the receptors are activated (Reul and Holsboer, 2002). It has also been suggested that CRFR2 may have actions that oppose or modulate responses mediated through CRFR1 (Reyes *et al.*, 2001; Grammatopoulos and Chrousos, 2002).

CRF, the first of its peptide family to be discovered (Vale *et al.*, 1983), has many behavioral and physiological effects. In addition to increasing anxiety-like responses, arousal, and HPA axis activation, numerous studies demonstrate extrahypothalamic actions of CRF in various fear conditioning paradigms (Steckler and Holsboer, 1999; Radulovic *et al.*, 1999b; Takahashi, 2001) and in learning and memory tasks such as visual discrimination, spatial learning, and inhibitory avoidance (Eckart *et al.*, 1999; Landgraf, 2001; Dautzenberg and Hauger, 2002; Eckart *et al.*, 2002; Grammatopoulos and Chrousos, 2002). Urocortin is the second endogenous peptide in the CRF ligand family to be discovered in mammals. It gets its name from the fish CRF-like peptide urotensin I (hence “uro” - 63% sequence similarity) and mammalian CRF (hence “cort” from corticotropin - 45% similarity) (Koob and Heinrichs, 1999), and it has behavioral effects similar to those of CRF (Eckart *et al.*, 1999). In the brain, CRF mRNA can be found in the anterior commissure, hypothalamus (median eminence, Pa, and periventricular nucleus in high concentrations), the limbic system (including the amygdala with high concentrations in the central (Ce) and cortical nuclei, BST, medial and lateral septum, and low concentrations in the hippocampus), and some brainstem nuclei (Chappell *et al.*, 1986; Eckart *et al.*, 1999; Eckart *et al.*, 2002). UCN-immunoreactivity can be found in the LS and hypothalamus (including low concentrations in the Pa and supraoptic nucleus) (Eckart *et al.*, 1999; Eckart *et al.*, 2002; Reul and Holsboer, 2002). CRF (rat/human, r/h, and ovine, o) and UCN bind with high affinity to CRFR1, and r/hCRF and UCN will both bind to CRFR2 with equal or less affinity than to CRFR1 (Dautzenberg and Hauger, 2002; Eckart *et al.*, 2002; Grammatopoulos and Chrousos, 2002; Reul and Holsboer, 2002).

A third member of the CRF ligand family, urocortin II (UCNII), shows high affinity for the CRFR2 receptor and little affinity for CRFR1 (Reyes *et al.*, 2001; Dautzenberg and Hauger, 2002). It is distributed primarily in subcortical areas such as the Pa, supraoptic and arcuate nuclei of the hypothalamus, locus coeruleus (LC), and some motor nuclei of the brainstem (Reyes *et al.*, 2001; Eckart *et al.*, 2002; Reul and Holsboer, 2002). Administration of UCNII causes Fos activation in the BST, Ce, Pa, parabrachial nucleus, and nucleus of the solitary tract. UCNII causes suppression of food intake (Reyes *et al.*, 2001), and some studies report anxiolytic effects of UCNII administration (Valdez *et al.*, 2002). As is the case with CRFR2 receptors, the role that UCNII plays in anxiety and stress is still not completely understood. Urocortin III (UCNIII), the most recently discovered mammalian ligand in the CRF family, is 40% homologous to UCNII and is distantly related to r/hCRF and human or mouse UCN. It displays lower affinity for CRFR2 than does UCNII and has no affinity for CRFR1 (Lewis *et al.*, 2001). UCNIII mRNA expression can be found in the AH, LH, LS, BST, the medial amygdala, and in some brainstem nuclei (Lewis *et al.*, 2001; Eckart *et al.*, 2002; Reul and Holsboer, 2002). Because of its distribution in brain sites associated with stress-responding, it is likely that UCNIII is involved in controlling behavioral and endocrine responses to stress (Lewis *et al.*, 2001). Discovery of these specific ligands for CRFR1 and CRFR2 has provided an opportunity to pharmacologically differentiate the roles of each receptor subtype in various behaviors.

Corticotropin Releasing Factor and Stress

There are numerous examples of stress-induced changes in CRF peptide or immunoreactivity. Acute stress decreases CRF in the median eminence and increases CRF in the LC, BST, and Pa (Chappell *et al.*, 1986; Watts, 1996; Hatalski *et al.*, 1998; Hsu *et al.*,

1998; Helmreich *et al.*, 1999; Jezova *et al.*, 1999; Hsu *et al.*, 2001). Stress-induced changes in CRF occur in the Ce, as well, though the type of stressor used may affect whether CRF increases or decreases post-stress (Hsu *et al.*, 1998). An increase in Ce CRF has been seen following acute restraint stress or footshock (Watts, 1996; Hsu *et al.*, 1998), but some studies using different types of stressors, such as osmotic stimulation or repeated restraint stress, have observed decreases (Watts, 1992) or no change, respectively, (Hatalski *et al.*, 1998) in Ce CRF post-stress. Stress can also affect how a CRF agonist modulates behavior. CRF or UCN has activational effects on behavior such as exploration in a non-stressful environment (like a familiar environment) but has suppressive effects on locomotor or exploratory behavior in a stressful environment (like a novel environment) (Koob and Heinrichs, 1999). Finally, stress has been reported to affect CRFR density and binding. Chronic social stress in tree shrews downregulates CRFR1 in brain regions involved in HPA axis control. Additionally, CRFR1 was upregulated in areas including the Cg, Ce, and La, though binding affinity was reduced in some of these areas (Fuchs and Flugge, 1995).

The time course for stress-induced changes in CRF can vary depending on the location in the brain where the changes are occurring. Changes can take place in minutes or not for hours. For example, CRF transcription increases in the Pa within about 5 minutes post-stress (Kovacs and Sawchenko, 1996). Further, changes in Pa CRF are usually only detectable immediately post-stress; however, changes in thalamic CRF can be seen 3 hours after restraint stress but not immediately afterwards (Hsu *et al.*, 2001). In other words, CRF transcription occurs quickly in brain regions involved in immediate responses to stress (like in the Pa to activate the HPA axis), while changes related to other functions (such as the modulation of learning and memory) may occur more slowly.

Corticotropin Releasing Factor as a Modulator of Fear and Anxiety

There are numerous studies demonstrating extrahypothalamic actions of CRF in various fear conditioning paradigms. For example, blockade of CRFR1 in rats reduces conditioned freezing, blocks fear potentiated startle, and decreases conditioned ultrasonic vocalizations (Steckler and Holsboer, 1999; Takahashi, 2001). It has also been demonstrated that three different CRF peptides (r/hCRF, oCRF, and rat UCN) dose-dependently enhance both tone- and context-fear conditioning in mice (Radulovic *et al.*, 1999b). However, there are exceptions to CRF's enhancing effects on fear conditioning. For example, unlike the results seen by Radulovic, et al. (Radulovic *et al.*, 1999b) with CRF peptides, transgenic overproduction of CRF in mice does not affect context-dependent conditioning to a shock, suggesting that different types of fear conditioning may be differentially modulated by CRF (van Gaalen *et al.*, 2002).

CRF is well-documented as a modulator of anxiety. CRFR1 antagonists reduce anxiety-like responses in tests such as CRF-induced startle, elevated plus maze, defensive withdrawal, and light-dark box exploration (Takahashi, 2001). Additionally, urocortin increases anxiety-like behavior in the social interaction test, an effect that is blocked by administration of the CRFR1 antagonist NBI3b1996 (Gehlert *et al.*, 2005). Further, mice overproducing CRF show increased anxiety-like behavior in the light-dark box test (van Gaalen *et al.*, 2002). CRF has also been shown to increase anxiety-like responses in CRF-potentiated startle via the BST, which, as described earlier, appears to be particularly associated with anxiety or unconditioned fear (Walker and Davis, 1997; Lee and Davis, 1997; Davis, 1998). For these reasons CRFR1 antagonists are being investigated as potential

drug therapies for anxiety disorders (Grammatopoulos and Chrousos, 2002; Reul and Holsboer, 2002).

Syrian Hamsters and Conditioned Defeat

As stated above, territorial aggression is a common situation in which an animal will encounter social conflict. Syrian hamsters (*Mesocricetus auratus*) are thought to be solitary animals that readily defend home territories (Nowack and Paradiso, 1983). When hamsters are singly housed in a laboratory, they will routinely attack intruders placed into their home cages (resident-intruder model), particularly if the resident is larger than its intruder. A dominant-subordinate (or “winner-loser”) relationship is formed quickly (Lerwill and Makings, 1971), even though the severity of the encounters in terms of biting or wounding is usually low. During a defeat, one hamster (usually the resident in a resident-intruder model) will initiate an attack, which includes pursuit of the other hamster and “biting.” Biting is usually directed to the flank of the opponent, but the skin is rarely broken during this action. During this time the other hamster may bite back, but usually it will quickly begin to display defensive and submissive behaviors. The dominant hamster may make additional attacks. Finally, defeat is determined when the previously attacked hamster flees or shows submissive/defensive behaviors without the dominant hamster making an attack. When a Syrian hamster is defeated in an agonistic encounter, it will subsequently fail to display any territorial aggression in future social encounters, even in its home cage in the presence of a non-aggressive, non-threatening, smaller intruder (Potegal *et al.*, 1993). This change in social behavior is termed conditioned defeat.

Conditioned defeat in hamsters may be a valuable model with which to study stress-responsive behavior. It can be reliably induced by a series of four, five-minute defeats or by

an acute 15-minute defeat (Huhman *et al.*, 1991; Potegal *et al.*, 1993; Jasnow and Huhman, 2001). When hamsters are defeated in this way, they subsequently show submissive behaviors such as flight, tail lift, tooth chatter, as well as defensive postures in the presence of a non-aggressive intruder (Potegal *et al.*, 1993; Jasnow *et al.*, 1999). A similar change in behavior has been observed in defeated rats and mice (van de Poll *et al.*, 1982; Frischknecht *et al.*, 1982). However, hamsters are a more desirable species to use because rats are social animals that usually show low levels of aggressive behavior unless they are housed in complex social situations, and aggressive strains of mice often have unacceptably high levels of aggression and injury.

Defeated hamsters exhibit increased plasma adrenocorticotrophic hormone (ACTH) and cortisol (CORT) concentrations (Huhman *et al.*, 1990; Huhman *et al.*, 1991; Huhman *et al.*, 1992), whereas dominant hamsters do not show this response. Additionally, CRF receptor (CRFR) antagonists reduce the expression of conditioned defeat as well as ACTH release following conditioned defeat testing (Jasnow *et al.*, 1999). The behavioral effect of acute defeat in hamsters is profound, and the physiological response consistently occurs and is unique to the defeated hamster. These effects can be obtained easily with a single exposure to social defeat, and the response is long lasting (Huhman *et al.*, 2003). Further, the behavioral and physiological affects listed here occur in virtually all hamsters, eliminating the need for prescreening. Because of the ease and consistency with which CD can be induced, as well as the fact that CD is a more ethologically relevant model than other artificial stressors (see above), conditioned defeat is a valuable model with which to study stress-responsive behavior.

Neurobiology of Conditioned Defeat

So far little is known about the circuitry controlling conditioned defeat. However, a series of studies have shown that the amygdala and the BST are both important for CD. Inactivation of the amygdala with the GABA agonist muscimol blocks both the acquisition and the expression of CD (Jasnow and Huhman, 2001), and blocking NMDA receptors in the amygdala with the antagonist AP5 blocks both acquisition and expression, as well (Jasnow *et al.*, 2004a). Involvement of CRF receptors has also been shown. ICV or intra-BST injection of the non-selective CRFR antagonist D-Phe CRF₍₁₂₋₄₁₎ (D-Phe) or the CRFR2-specific antagonist antisauvagine-30 (ASVG) reduces the acquisition and expression of CD (Jasnow *et al.*, 1999; Jasnow *et al.*, 2004b; Cooper and Huhman, 2005a; Cooper and Huhman, 2005b). Additionally, a combination of unilateral Ce lesion with contralateral intra-BST injection of D-Phe CRF (thereby interrupting what would be ipsilateral “communication” between the Ce and the BST) reduces the expression of CD. These results suggest that projections from the Ce to the BST are involved in the expression of CD. It is already known that there are projections from the Ce to the BST that release CRF (Sakanaka *et al.*, 1986). Because CRFR antagonism in the BST reduces CD, it is likely that the projections from the Ce to the BST that are involved in CD are releasing CRF.

Scope of this Dissertation

Though there is strong evidence that the amygdala and BST are part of the neurocircuitry mediating CD and that extrahypothalamic CRF transmission is important in this circuitry, there is little else known about this system or what role CRF plays to modulate CD and at which receptor subtypes. The goal of this project is to investigate the role of CRF type 1 and 2 receptors in CD and to identify neuroanatomical locations where CRF may be

acting. Specific Aim 1 will test the hypothesis that CRF receptors modulate the expression of conditioned defeat by answering the following question: will a ventricular injection of CRF enhance the acquisition or expression of conditioned defeat after a suboptimal defeat encounter, and would this effect be due to cortisol release via HPA axis activity or central CRF transmission? Specific Aim 2 will test the hypothesis that CRFR2, and not CRFR1, is important in mediating the effects of CRF on conditioned defeat by answering the following question: will CRFR1 or CRFR2 agonists given before testing enhance the expression of conditioned defeat? Finally, Specific Aim 3 will use receptor autoradiography to test the hypothesis that CRFR2 receptor binding is increased following defeat by answering the following questions: 1) how are CRFR1 and CRFR2 distributed throughout stress circuits in hamster brain, particularly in the LS, amygdala, BST, and hypothalamus, and 2) are there differences in CRF receptor densities between defeated and non-defeated hamsters, and are those differences reflective of changes in CRFR1 or CRFR2 receptor densities?

Central Corticotropin Releasing Factor Modulates the Expression of Agonistic Behavior in Syrian Hamsters

Alicia N. Faruzzi^{1,2}, Matthew A. Cooper¹, and Kim L. Huhman^{1*}

¹Center for Behavioral Neuroscience, Department of Psychology, ²Brains & Behavior Program, Georgia State University, Atlanta, GA, USA

*To whom correspondence should be addressed:

Kim L. Huhman
Center for Behavioral Neuroscience
Georgia State University
P.O. Box 3966
Atlanta GA 30302-3966
Phone: 404-651-1636
Fax: 404-651-3929
khuhman@gsu.edu

Running title: CRF and agonistic behavior in hamsters

Summary

Social conflict is a biologically relevant stressor that can affect physiological and psychological markers of stress. When a Syrian hamster loses an agonistic encounter, it shows elevated plasma adrenocorticotrophic hormone (ACTH) and cortisol (CORT). A defeated hamster also fails to display normal territorial aggression and instead displays submissive behavior in the presence of a non-aggressive intruder, a behavioral change termed conditioned defeat (CD). Previous studies in our laboratory have shown that blockade of corticotropin releasing factor (CRF) receptors attenuates the expression of CD. Because CRF is a critical component of the hypothalamo-pituitary-adrenal axis (HPA) response to stress, it is possible that the observed increases in CORT following defeat provide feedback important for the formation of CD. Previous studies in our laboratory have shown that blocking ACTH release with a specific CRF type 1 receptor antagonist does not reduce the expression of CD, however, suggesting that CRF effects are central and not peripheral. The purpose of the present study was to further elucidate the role of CRF in CD. In Experiment 1, the glucocorticoid synthesis inhibitor metyrapone was administered SC to hamsters before CD training or testing to determine whether CORT affects either the acquisition or expression of CD. Metyrapone administration had no effect on CD, though it did successfully block elevations in plasma CORT following defeat. In Experiment 2, hamsters received a microinjection of rat/human CRF into the lateral ventricle prior to CD training or testing to determine whether central CRF administration enhances CD acquisition or expression after a suboptimal defeat training session. Hamsters given CRF before CD testing exhibited significant increases in submissive behavior compared to hamsters receiving vehicle. CRF administration also increased aggressive behavior in non-defeated hamsters.

Collectively, these data suggest that CRF modulates CD via direct neurotropic action. Further, it appears that CRF has different effects on agonistic behavior depending on the hamster's previous social experience.

Keywords: glucocorticoids, stress, conditioned defeat, metyrapone, fear conditioning, aggression

1. Introduction

Stressful life events are linked to an increased incidence of psychopathology, including affective and anxiety disorders (Holsboer, 1988; Barden, 1996; Plotsky *et al.*, 1998; Mitchell, 1998; Arborelius *et al.*, 1999). In order to elucidate how stress can lead to psychological disorders, and to identify possible pharmacological therapies for those disorders, various models of stress have been developed. Social conflict is an inevitable part of life in many species and thus is a more biologically relevant stressor than are other stressors commonly used in the laboratory, such as restraint or footshock. When conflict arises, the outcome (i.e., whether the individual “wins” or “loses”) has a strong and lasting impact. Losers of agonistic encounters, but not winners, exhibit increased heart rate, elevated blood pressure, and suppressed immune function (Bohus *et al.*, 1993; Blanchard *et al.*, 1995; Meehan *et al.*, 1995), as well as activation of the hypothalamo-pituitary-adrenal (HPA) axis (Pich *et al.*, 1993; Buwalda *et al.*, 1999; Jasnow *et al.*, 2001). Defeated animals also exhibit many behavioral changes, such as decreased reproductive, exploratory, or aggressive behavior, decreased food and water intake, and increased anxiety (Heinrichs *et al.*, 1992; Potegal *et al.*, 1993; Rodgers and Cole, 1993; Albonetti and Farabollini, 1994). These physiological and behavioral effects can be used as targets for pharmacological or other manipulations to identify specific brain regions, neurochemical signals, or molecular changes that are important in the development of stress-induced psychological disorders.

Syrian hamsters (*Mesocricetus auratus*) are thought to be solitary animals that readily defend their home territories (Nowack and Paradiso, 1983). When hamsters are singly housed in a laboratory, they routinely attack intruders placed into their home cages (resident-intruder model), particularly if the resident is larger than the intruder. A dominant-

subordinate (or “winner-loser”) relationship is formed quickly (Lerwill and Makings, 1971), even though the severity of the encounter between hamsters (i.e., biting or wounding) is usually low. After a male hamster is defeated in an agonistic encounter, it will subsequently fail to display any territorial aggression in future social encounters and will instead show increased submissive or defensive behavior, even when these encounters occur in its own home cage against a smaller, non-aggressive intruder (Potegal *et al.*, 1993). This change in social behavior is termed conditioned defeat (CD). In addition to this striking behavioral change, defeated hamsters exhibit increased plasma adrenocorticotrophic hormone (ACTH) and cortisol (CORT) concentrations following a social encounter (Huhman *et al.*, 1990; Huhman *et al.*, 1991; Huhman *et al.*, 1992), whereas dominant hamsters do not.

Corticotropin releasing factor (CRF) is a 41-amino acid peptide (Vale *et al.*, 1983) located in the anterior pituitary and throughout the brain (Chappell *et al.*, 1986; Eckart *et al.*, 1999; Eckart *et al.*, 2002). It has many behavioral and physiological effects, including increased anxiety-like responding, increased arousal and HPA axis activation, and enhancement of learning and memory in tasks such as visual discrimination, spatial learning, inhibitory avoidance, and fear conditioning (Eckart *et al.*, 1999; Landgraf, 2001; Dautzenberg and Hauger, 2002; Eckart *et al.*, 2002; Grammatopoulos and Chrousos, 2002). CRF is a critical component of the endocrine response to stress. Following exposure to a stressful event, CRF is released from the parvicellular portion of the paraventricular nucleus (Pa) and it triggers release of ACTH from anterior pituitary corticotrophes. ACTH, in turn, stimulates adrenocortical release of glucocorticoids, which has a number of effects including gluconeogenesis to provide energy (to sustain the “flight or flight” response) and reduced inflammation. Glucocorticoids also provide feedback to the brain to control the HPA axis

response to stress. In addition to the critical role of CRF in the HPA axis response to stress, CRF peptides and receptors are distributed throughout the brain (Bittencourt and Sawchenko, 2000; Lewis *et al.*, 2001; Reyes *et al.*, 2001; Dautzenberg and Hauger, 2002; Eckart *et al.*, 2002; Reul and Holsboer, 2002), suggesting a wider role for this peptide. Consistent with this view, numerous studies demonstrate extrahypothalamic actions of CRF in various fear conditioning paradigms (Steckler and Holsboer, 1999; Radulovic *et al.*, 1999b; Takahashi, 2001).

CRF may modulate agonistic behavior by its regulation of glucocorticoids. Acute increases in glucocorticoids have been shown to increase aggression or submission depending on the social context (Leshner, 1980; Leshner, 1983). Glucocorticoids might produce these variable effects by acting on brain mechanisms to increase general arousal and thereby increase the salience of incoming stimuli. More recently, glucocorticoids have been proposed to directly modulate ongoing aggressive behavior through rapid effects on neurotransmitter systems (Haller *et al.*, 1998; Mikics *et al.*, 2004). Glucocorticoids might also affect future agonistic behavior by modulating memory of the social interaction. Increased glucocorticoids produced by an acute stressful experience have been shown to facilitate the acquisition of some associative memory tasks (Beylin and Shors, 2003). Likewise, administration of corticosterone and glucocorticoid receptor agonists have been shown to enhance the consolidation of memories for emotionally arousing experiences (Buchanan and Lovallo, 2001; Roozendaal *et al.*, 2001; Hui *et al.*, 2004). Also, the suppression of glucocorticoid synthesis with metyrapone impairs memory consolidation on emotionally motivated tasks such as a water maze and inhibitory avoidance (Roozendaal *et al.*, 1996; Liu *et al.*, 1999). The basolateral amygdala (BLA) is the primary brain region that

mediates the effects of stress and glucocorticoids on memory consolidation and retrieval (Roozendaal and McGaugh, 1997; Roozendaal *et al.*, 2001; Roozendaal *et al.*, 2002; Roozendaal *et al.*, 2004).

Previous work in our laboratory suggests that CD is modulated by CRF. D-Phe CRF₍₁₂₋₄₁₎, a non-selective CRF receptor antagonist, reduces CD, whereas CP-154,526, a selective CRF 1 receptor antagonist, blocks the plasma ACTH response to defeat but does not reduce the display of submissive behavior (Jasnow *et al.*, 1999). This result suggests that CRF is acting via a central and not a peripheral mechanism. On the other hand, these data suggest that adrenocorticoids can have a pronounced effect on behavior, including agonistic behavior. One goal of the present study was to determine whether CRF modulates CD by acting centrally, peripherally, or both. Based on the growing literature showing effects of glucocorticoids on agonistic behavior, as well as learning and memory, it remains plausible that administration of the glucocorticoid synthesis inhibitor metyrapone might reduce the acquisition or expression of CD. In addition, we hypothesized that intracerebroventricular injection of CRF would enhance the acquisition as well as the expression of CD following a suboptimal defeat.

2. Materials and Methods

Subjects

Male Syrian hamsters were purchased from Charles River Laboratories. Experimental animals were three to four months old and weighed 120-140 g at the start of the study. The animals were individually housed for 10 days to two weeks prior to testing in a temperature controlled ($20^{\circ}\text{C} \pm 2^{\circ}$) colony room and were maintained on a 14:10h light:dark cycle. Older animals that weighed 160-180g were housed individually and used as resident

aggressors during the defeat phase of the CD protocol. Younger animals (two months) that weighed 100-110g were group-housed (five animals per cage) and were used as non-aggressive intruders during the testing phase. All animals were housed in polycarbonate cages (20 x 40 x 20 cm) with corncob and cotton bedding materials and wire mesh tops. Food and water were available ad libitum. The cages of experimental animals and resident aggressors were not changed for at least one week prior to testing. All training or testing occurred within the first three hours after the onset of the dark phase of the light:dark cycle in order to minimize circadian variation of the dependent measures. All experimental animals were handled daily for at least one week prior to the beginning of any experiment in order to habituate them to the stress of being handled by the experimenter. During the experiment if any animal was bitten such that it bled, the encounter was stopped and the animal was examined and treated immediately. All procedures and protocols were approved by the Georgia State University Institutional Animal Care and Use Committee.

Experiment 1a

To test metyrapone's effectiveness at blocking increased CORT release following defeat, 18 animals were injected (SC) with metyrapone (50 mg/kg or 100 mg/kg in 0.2 ml vehicle) or vehicle 90 min prior to a 15-min training session with a resident aggressor. Metyrapone [2-methyl-1,2-di-3pyridyl-1-propanone (Sigma)] was dissolved in polyethylene glycol and diluted with saline to reach a final concentration of 40% polyethylene glycol. Drug doses and delay between injection and testing were selected based on previous research (Roozendaal *et al.*, 1996; Liu *et al.*, 1999). Immediately following social defeat, hamsters were rapidly decapitated and trunk blood was collected for CORT radioimmunoassay (RIA). Trunk blood was transferred to heparinized tubes kept on ice, centrifuged at 4°C and 3500

rpm for 20 min, and the plasma was stored at -20°C until assay. Total plasma cortisol was measured in duplicate samples at the Endocrine Core Laboratory, Yerkes Primate Research Center of Emory University, using an RIA kit produced by Diagnostic Systems Laboratories (Webster, TX). The normal range for this assay is 0.5 – 60 µg/dl in a 25µl dose. Intraassay variation was 4.9%, and all samples were run together.

Experiment 1b

Experiment 1b was designed to test the effect of metyrapone on the acquisition and expression of CD. To investigate effects on acquisition, 20 adult male Syrian hamsters were given metyrapone (50 mg/kg) or vehicle 90 min prior to a 15-min CD training session with a resident aggressor. To investigate effects on expression, 18 adult male hamsters were given metyrapone (50 mg/kg) or vehicle 90 min prior to a 5-min CD test.

The conditioned defeat model has been described elsewhere (Jasnow and Huhman, 2001) and is briefly described here. Prior to each experiment, the animals were matched by weight and randomly assigned to groups. On the training day, experimental animals were transported to a testing room. Training consisted of one 15-min exposure to a resident aggressor in the aggressor's home cage. The resident aggressor reliably attacked the experimental animal, and all of the experimental animals displayed high levels of submissive and defensive behavior during training. To correct for the total duration of defeat, exposure time began following the first attack by the resident aggressor (which usually occurred within the first 30 sec of the training trial). Twenty-four hours following defeat, experimental animals were tested for CD. The animals were transported to the testing room and a non-aggressive intruder was placed in their home cage for 5 min. Total durations of submissive,

aggressive, social, and non-social behaviors were recorded during testing sessions (see *Behavioral Scoring and Analysis*).

Experiment 2

Experiment 2 was designed to test the hypothesis that ICV injection of CRF would enhance the acquisition and expression of CD following a suboptimal defeat training session. A single 5-min defeat session has been shown previously to elicit very low levels of submissive behavior in male Syrian hamsters when they are tested 24h later (Jasnow *et al.*, 2002). Therefore, a 5-min defeat session is considered suboptimal for inducing CD and provides the opportunity to observe a pharmacological enhancement of CD. Hamsters were deeply anesthetized with sodium pentobarbital (90 mg/kg) and placed in a stereotaxic instrument. The skull was leveled between lambda and bregma prior to implantation of a guide cannula. Hamsters were then implanted unilaterally with a 4 mm, 26-gauge guide cannula aimed at the lateral ventricle (LV). Final stereotaxic coordinates were 0.8 mm anterior and 1.1 mm lateral to bregma. The injection cannula (33-gauge) extended 1.1 mm beyond the guide to reach the final depth of -3.4 mm below dura. Dummy stylets were placed in the guide cannulae in order to keep them unobstructed. Hamsters were allowed at least one week of recovery before commencing behavioral experiments.

To test for effects on acquisition, hamsters received injection of either vehicle or rat/human CRF (r/hCRF; 0.2 nmol) 40 mins prior to a 5-min defeat training session as briefly described above and elsewhere (Jasnow and Huhman, 2001). To test for effects on expression of CD, a different group of hamsters received either vehicle or r/hCRF (0.2 nmol) 40 mins prior to testing for CD (24h following defeat training). Testing for CD occurred as described in Experiment 1. Because r/hCRF has been reported to have anxiogenic effects

(Takahashi *et al.*, 1989; Owens and Nemeroff, 1993; Weiss *et al.*, 1994; Heinrichs *et al.*, 1997; Holsboer, 1999; Steckler and Holsboer, 1999; Arborelius *et al.*, 1999; Eckart *et al.*, 1999; Radulovic *et al.*, 1999a; Takahashi, 2001), a no-defeat control group was included in Experiment 2 to control for a main effect of r/hCRF on behavior. One third of the hamsters were exposed to a resident aggressor's empty cage for 5 min in lieu of being defeated, and drug was administered as described above. At the conclusion of testing, hamsters were sacrificed and the brains collected for verification of the injection site.

CRF (rat/human CRF; Sigma) was solubilized in sterile saline (0.9%). Hamsters received either 0.0 or 0.2 nmol r/hCRF into the LV. This dose was determined from a dose-response curve obtained in our laboratory using either 0.0, 0.2 or 2.0 nmol r/hCRF (Faruzzi and Huhman, 2001). Microinjections were made with a 5.0 μ l Hamilton syringe connected to the injection cannula via polyethylene tubing. A final volume of 3.0 μ l was injected over the course of 1 min, and the injection cannula was left in place for an additional 1 min to allow for drug diffusion. Hamsters were allowed to move freely during injections.

Following testing, each hamster was given a lethal dose of sodium pentobarbital. A volume of 0.5 μ l of ink was microinjected using the same length injection needle as was used to administer r/hCRF, and the brains were removed and stored in 10% formalin for at least 24h. A razor blade was used to slice through the LV. A hit was determined by the presence of ink in the LV and a miss was assigned if the ink was primarily located above the lateral ventricles or in another location. Any animal that was assigned a miss was not used in statistical analysis.

Behavioral Scoring and Analysis

All testing sessions were recorded and later scored by an observer blind to the experimental conditions using Noldus Observer v. 5.0 (Noldus Information Technology, Wageningen, Netherlands). Total duration of four classes of behavior were scored during each 5 min test: 1) Submissive: flee, avoid, tail up, upright and side defense, full submissive posture, stretch-attend, head flag, attempted escape from the cage; 2) Aggressive: upright and side offense, chase, and attack (including bite); 3) Social: attend, approach, investigate, sniff, nose touching, and flank marking; 4) Nonsocial: locomotion/exploration, self-groom, nesting, feeding, and sleeping. A subset of sessions was scored by a second observer, and inter-observer reliability on the total duration of submissive behavior was >90%. When drug was administered prior to social defeat, training sessions were recorded as well and scored for the total duration of agonistic behavior.

The total durations of submissive, aggressive, social, and non-social behavior were individually analyzed using T-tests or one-way between-subjects analyses of variance (ANOVAs) with drug treatment as the between-subjects factor. Tukey and Least Significant Difference (LSD) post hoc tests were used for pairwise comparisons. Nonparametric statistics (Mann-Whitney U tests) were used for any comparisons with a significant Levene's test of homogeneity of variances. Planned comparisons were used to analyze Experiment 2 data. Alpha was set at $p < 0.05$.

3. Results

Experiment 1

Metypapone effectively blocked the increase in cortisol generally observed after social defeat ($F(2,15)=40.5$, $p < 0.001$, Tukey tests $p < 0.001$; Figure 1). In subsequent

experiments, we used the lowest effective dose only (50 mg/kg). When given prior to social defeat, however, this dose of metyrapone did not significantly alter submissive behavior during testing ($t(17)=0.03$, $p>0.05$; Figure 2a). In addition, metyrapone given before training did not significantly alter aggressive, social, or nonsocial behavior during testing (T-tests, $p>0.05$). One animal was excluded from the study because it was not defeated by the resident aggressor. Also, when metyrapone was given prior to testing, it did not significantly alter the expression of submissive behavior ($t(16)=0.97$, $p>0.05$; Figure 2b) or aggressive, social, or nonsocial behaviors (T-tests, $p>0.05$).

Experiment 2

Analysis using planned comparisons (defeat-CRF vs. defeat-vehicle and no-defeat-CRF vs. defeat-CRF) revealed that ICV r/hCRF significantly enhanced the expression of CD (Figure 3). Defeated hamsters that received r/hCRF prior to testing displayed significantly more submissive behavior when tested with a non-aggressive intruder than did non-defeated hamsters receiving r/hCRF or defeated hamsters receiving vehicle, $F(1,9)=13.29$ and $F(1,8)=19.79$, respectively, $p<0.05$. Defeated hamsters that received r/hCRF also showed significantly less nonsocial behavior during testing than did defeated hamsters that received vehicle ($F(1,8)=6.11$, $p<0.05$). However, further analysis revealed no differences in the types of nonsocial behaviors displayed by defeated hamsters receiving r/hCRF compared to defeated controls. These hamsters displayed normal exploratory behavior for the majority of the 5-min test. Finally, non-defeated hamsters receiving r/hCRF prior to testing displayed significantly more aggressive behavior than did defeated hamsters receiving r/hCRF (Mann-Whitney U test, $Z=-2.30$, $p<0.05$). There was no effect of r/hCRF on the acquisition of CD

($F(1,20)=0.06$ and $F(1,24)=0.16$, $p>0.05$) or on aggressive behavior (Mann-Whitney U test, $Z=-0.84$ and $F(1,24)=0.14$, $p>0.05$).

4. Discussion

Collectively, the data presented here indicate that CRF modulates the expression, but not the acquisition of CD, and that this effect is not mediated by the HPA axis. In the current study, administration of r/hCRF altered the expression of CD while blockade of glucocorticoid synthesis with metyrapone did not. This finding is consistent with previous data from our laboratory showing that blockade of CRF type 1 receptors significantly attenuates the HPA axis response in defeated hamsters but does not affect CD (Jasnow *et al.*, 1999). Therefore, it would appear that CRF modulates CD through central circuitry and does not affect CD via the peripheral hormonal stress response.

We also observed a significant increase in aggressive behavior in non-defeated hamsters that received r/hCRF prior to testing, suggesting that CRF's activational effects on agonistic behavior are dependent on the previous social experience of the hamster. Thus, defeated hamsters receiving r/hCRF show increases in submissive behavior while non-defeated hamsters exhibit increases in aggressive behavior. There are two important implications of these data. First, CRF appears to affect agonistic behavior broadly and not merely defensive or fearful responses as was suggested from previous studies examining CD (Jasnow *et al.*, 1999). Second, these data strongly suggest that there are separate neural mechanisms controlling opposing aspects of agonistic behavior. If all dominant and subordinate behavior was controlled by the same neural circuit, then it would be expected that manipulations that decrease one behavior would necessarily increase the opposing behavior. However, these and previous data (Jasnow *et al.*, 1999; Jasnow and Huhman,

2001; Jasnow *et al.*, 2004a) demonstrate that, although increases in submissive behavior are accompanied with decreases in aggressive behavior, a decrease in or the absence of submissive behavior is not necessarily accompanied by a return of territorial aggression. Pharmacological manipulations that decrease submission are instead associated with increased social or nonsocial behavior, collectively suggesting that subtypes of agonistic behavior are controlled by separate neural mechanisms. The fact that CRF may increase either submissive or aggressive behavior suggests that it can have activational effects on either aspect of agonistic behavior, but other factors (previous social experience, for example) determine which behavior will be expressed. Ongoing experiments in our laboratory are using additional control groups to investigate whether CRF is indeed increasing multiple aspects of agonistic behavior and not just submissive behavior. Additionally, we are examining whether these effects are specific to agonistic behavior or result from a state of generalized anxiety or increased locomotor behavior.

In many animals, social defeat results in increased levels of plasma glucocorticoids and decreased aggressive behavior (Brain, 1980; Sapolsky, 1990; Schuett *et al.*, 1996). In the present study, we showed that blocking glucocorticoid synthesis did not alter the acquisition or expression of CD. This result is consistent with previous research from our laboratory showing that blockade of plasma ACTH release fails to reduce CD (Jasnow *et al.*, 1999). Our results are also similar to those found for green anole lizards in which blocking CORT synthesis during exposure to an aggressive video did not alter future aggressive behavior to a novel challenger (Yang and Wilczynski, 2003). However, our results contrast with recent evidence showing that glucocorticoids facilitate the acquisition of stress-induced changes in behavior (Calvo *et al.*, 1998; Liu *et al.*, 1999; Cordero *et al.*, 2002). Recent

evidence also suggests that the elevation of glucocorticoids that occurs following social defeat modulates the development of aggression in Syrian hamsters (Wommack *et al.*, 2003). Our study suggests that the role for glucocorticoids in regulating behavioral responses to social defeat during puberty may not extend into adulthood. In sum, although an acute social defeat produces activation of the HPA axis, it appears that glucocorticoid feedback is not a critical part of the neural circuitry regulating CD.

CRF has previously been shown to modulate social learning in rats (Heinrichs, 2003). In this earlier study, the CRF antagonist D-Phe CRF impaired social learning and r/hCRF enhanced social learning when given prior to an initial social exposure. However, in the present study, the fact that there is an effect on expression only and not on the acquisition of conditioned defeat suggests that CRF may not have a role in the learning of CD. It is possible that CRF is involved in the retrieval of social memories, though it is also possible that CRF only has general motivational effects on agonistic behavior. Our lab does have preliminary data suggesting that CRF antagonists block the acquisition of CD, indicating that CRF may have a role in learning. More research is necessary to better understand the role CRF is playing in modulating agonistic behavior and conditioned defeat in hamsters.

In conclusion, the present study demonstrates that CRF has a direct neurotrophic effect on stress-responsive behavior in Syrian hamsters. Blockade of glucocorticoid synthesis with metyrapone blocks neither the acquisition nor expression of submissive behavior in previously defeated hamsters. On the other hand, blockade of CRF receptors reduces the expression submissive behavior (Jasnow *et al.*, 1999) and central administration of CRF enhances the expression of submissive behavior. The current study also demonstrates that CRF has a potent activational effect on agonistic behavior, but the type of

behavior that is affected depends on the animal's previous social experience. Further studies will elucidate the role of CRF receptors in conditioned defeat by examining which receptor subtype(s) is responsible for modulating submissive and aggressive behavior. It will also be important to determine where in the brain CRF is acting to modulate each aspect of agonistic behavior.

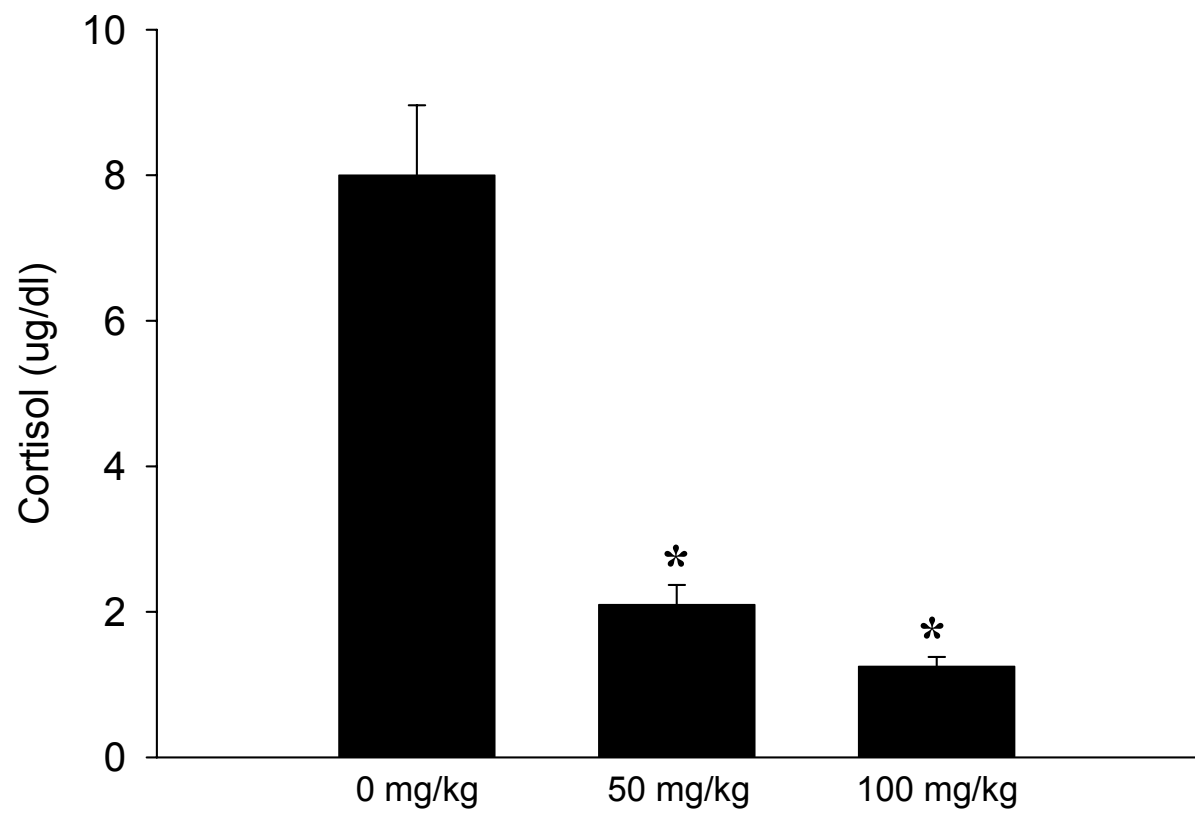
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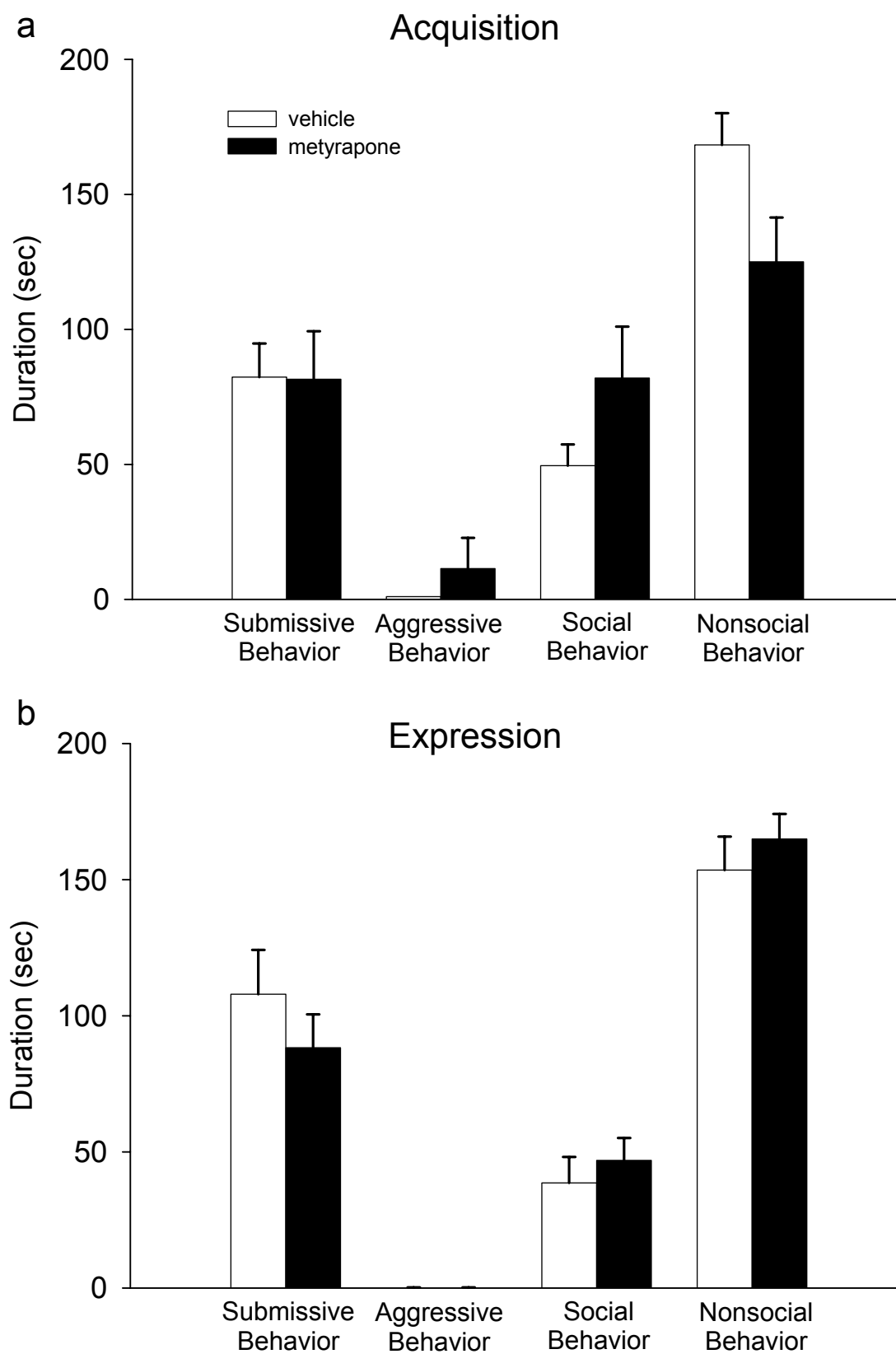
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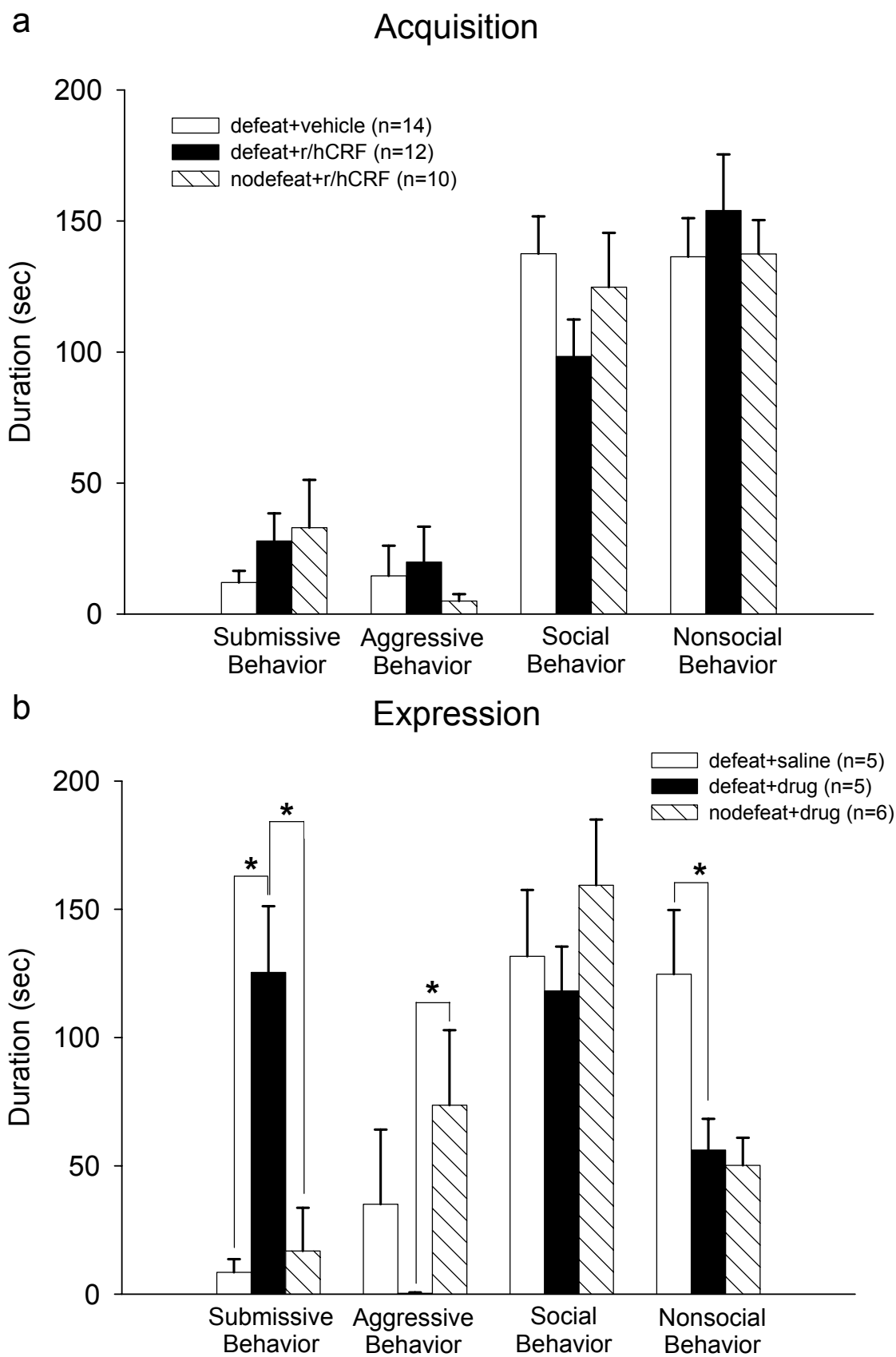
Figure 2.1. Means \pm SEM of cortisol in male hamsters receiving metyrapone (0.0, 50.0, 100 mg/kg SC). These data indicate that metyrapone significantly attenuated the cortisol response to a 15-min social defeat stressor. ($p < 0.05$)

Figure 2.2. Means \pm SEM of duration of submissive, aggressive, social and nonsocial behavior in male hamsters receiving metyrapone either a) before training (acquisition effects) or b) before CD testing (expression effects). ($p > 0.05$)

Figure 2.3. Means \pm SEM of duration of submissive, aggressive, social and nonsocial behavior in defeated and non-defeated male hamsters receiving r/hCRF either a) before training (acquisition effects) or b) before CD testing (expression effects). (planned comparisons: defeat-CRF vs. defeat-vehicle and no-defeat CRF vs. defeat-CRF; $*p < 0.05$)







Previous Social Experience Modulates the Effects of Corticotropin Releasing Factor on Agonistic Behavior in Syrian Hamsters

Alicia N. Faruzzi^{1,2*}, Lauren R. Zelinski¹, Jordan Tenenbaum¹, and Kim L. Huhman^{1,2}
¹Center for Behavioral Neuroscience, Department of Psychology, ²Brains & Behavior Program, Georgia State University, Atlanta, GA, USA

*To whom correspondence should be addressed:

Alicia N. Faruzzi
Center for Behavioral Neuroscience
Georgia State University
P.O. Box 3966
Atlanta GA 30302-3966
Phone: 404-463-9440
Fax: 404-651-3929
afaruzzi98@alumni.purdue.edu

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ABSTRACT

The non-specific corticotropin releasing factor receptor (CRFR) agonist rat/human CRF has previously been shown to enhance the expression of conditioned defeat (CD) in male Syrian hamsters. In addition, it has been shown that this agonist may increase the expression of aggressive behavior in previously undefeated hamsters, suggesting a general role for CRF in the expression of agonistic behavior. The current study further investigated the effects of CRF on agonistic behavior by examining which CRFR subtype is mediating these effects. In Experiment 1a, previously defeated and non-defeated hamsters received vehicle, ovine CRF (oCRF), a CRFR1-specific agonist, or human urocortin II (UCNII), a CRFR2-specific agonist, prior to testing with a non-aggressive intruder. oCRF significantly enhanced the expression of CD in defeated hamsters and showed a trend toward increased aggressive behavior in non-defeated hamsters, while UCNII had no effect. In Experiment 1b, previously defeated and non-defeated hamsters received vehicle or antisauvagine-30 (ASVG), a CRFR2-specific antagonist, in addition to receiving vehicle or oCRF prior to testing. Administration of ASVG did not block the replication of previously observed effects of oCRF on aggressive or submissive behavior, suggesting that oCRF is not working via spillover to CRFR2. Finally, Experiment 2 was designed to test whether the effects of oCRF observed in Experiment 1 were due to increases in anxiety-like behavior. The results showed no effect of ICV oCRF injection on open field or defensive withdrawal behavior in compared to vehicle hamsters. Overall, these results suggest that activation of CRFR1 has potent enhancing effects on the expression of agonistic behavior in Syrian hamsters. Further, this effect appears to be modulated by the hamster's previous social experience.

KEYWORDS: conditioned defeat, aggression, anxiety, stress, CRF receptors, antisauvagine, ovine CRF, urocortin II

INTRODUCTION

When a male Syrian hamster (*Mesocricetus auratus*) is defeated in an agonistic encounter, it will subsequently fail to display any territorial aggression in future social encounters. The hamster will instead show increased submissive or defensive behavior, even when these encounters occur in its own home cage against a smaller, non-aggressive intruder (Potegal *et al.*, 1993). This change in behavior is termed conditioned defeat (CD). In addition to this striking behavioral change, defeated hamsters exhibit increased plasma adrenocorticotrophic hormone (ACTH) and cortisol (CORT) concentrations following a social encounter (Huhman *et al.*, 1990; Huhman *et al.*, 1991; Huhman *et al.*, 1992), whereas dominant hamsters do not. These behavioral and physiological changes have led to an investigation of CD as both a model of stress and of fear conditioning. These investigations have suggested, among other things, a role for corticotropin releasing factor (CRF) in the expression and possibly the acquisition of CD.

CRF is a 41-amino acid peptide (Vale *et al.*, 1983) located in the anterior pituitary and throughout the brain (Chappell *et al.*, 1986; Eckart *et al.*, 1999; Eckart *et al.*, 2002). It has many behavioral and physiological effects, including increased anxiety-like responding, increased arousal and activation of the hypothalamo-pituitary-adrenal (HPA) axis. CRF is a critical component of the HPA axis response to stress, a response which has a number of effects including gluconeogenesis (to sustain the “flight or flight” response), reduced inflammation, as well as negative feedback to the brain to ultimately turn off the stress response. In addition to the critical role of CRF in the HPA axis response to stress, CRF peptides and receptors are distributed throughout the brain (Bittencourt and Sawchenko, 2000; Lewis *et al.*, 2001; Reyes *et al.*, 2001; Dautzenberg and Hauger, 2002; Eckart *et al.*,

2002; Reul and Holsboer, 2002), suggesting a wider role for this peptide. Consistent with this view, numerous studies demonstrate extrahypothalamic actions of CRF in various fear conditioning paradigms (Steckler and Holsboer, 1999; Radulovic *et al.*, 1999b; Takahashi, 2001) and in learning and memory tasks such as visual discrimination, spatial learning, and inhibitory avoidance (Eckart *et al.*, 1999; Landgraf, 2001; Dautzenberg and Hauger, 2002; Eckart *et al.*, 2002; Grammatopoulos and Chrousos, 2002).

Previous research in our laboratory (Jasnow *et al.*, 1999; Jasnow *et al.*, 2004b; Cooper and Huhman, 2005a) has indicated that nonselective CRF receptor (CRFR) antagonists can attenuate the expression of conditioned defeat. We have also shown that ICV administration of the nonselective receptor agonist rat/human CRF enhances CD in previously defeated hamsters while it enhances territorial aggression in hamsters that have not previously been defeated (A.N. Faruzzi, M.A. Cooper, and K.L. Huhman, submitted). In addition to the nonspecific CRF receptor antagonist D-Phe CRF (12-41), we have also shown that administration of the CRFR2-specific antagonist antisauvagine-30 attenuates the expression of CD (Jasnow *et al.*, 1999; Jasnow *et al.*, 2004b; Cooper and Huhman, 2005a), while the CRFR1 specific antagonist CP-154,526 does not (Jasnow *et al.*, 1999; Cooper and Huhman, 2005a). Although these results suggest that CRFR2, and not CRFR1, is modulating CD, investigations by other laboratories into the role of CRF in agonistic behavior have indicated a role for CRFR1. For example, the CRFR1 receptor antagonist antalarmin reduces defensive behavior in defeated mice (Robison *et al.*, 2004) and reduces anxious and fearful responses normally displayed when rhesus macaques are visually exposed to conspecifics (Habib *et al.*, 2000). Additionally, administration of the CRFR1 antagonist SSR125543A reduces territorial aggression in male Syrian hamsters (Farrokhi *et al.*, 2004). There is also

evidence that decreased CRFR2 binding reduces maternal aggression but does not affect intermale aggression in CRFR2-mutant mice (Gammie *et al.*, 2005), suggesting that CRFR2 does not play an important role in offensive aggression. Thus, while data from our lab have initially supported a more important role for the CRFR2 in the modulation of CD, other published data indicate that the CRFR1 may also have important effects on agonistic behavior. The purpose of the present study was to use specific CRF receptor agonists to investigate which CRF receptor mediates the reported effects of CRF on agonistic behavior in Syrian hamsters.

The standard training protocol for CD involves a 15-min defeat training session. A single 5-min defeat session elicits only very low levels of submissive behavior in male Syrian hamsters when they are tested 24h later (Jasnow *et al.*, 2002). Because a 5-min defeat session produces only a low level of CD, it provides the opportunity to observe a pharmacological enhancement of CD. Using this abbreviated defeat training, we have previously shown that ICV injection of r/hCRF enhances the expression of submissive behavior in hamsters (A.N. Faruzzi, M.A. Cooper, and K.L. Huhman, submitted). The current study used this design to test whether administration of ovine CRF (oCRF), a CRFR1 specific agonist (Dautzenberg and Hauger, 2002), or urocortin II (UCNII), a CRFR2 specific agonist (Reyes *et al.*, 2001; Jahn *et al.*, 2004), would enhance the expression of conditioned defeat. This study also tested whether oCRF or UCNII would enhance the expression of aggressive behavior in hamsters that had not previously experienced social defeat. Finally, because CRFR1 binding is associated with increases in anxiety-like behavior (Heinrichs *et al.*, 1997; Smith *et al.*, 1998; Liebsch *et al.*, 1999; Radulovic *et al.*, 1999b; Takahashi, 2001; Zorrilla *et al.*, 2002; van Gaalen *et al.*, 2002; Spina *et al.*, 2002; Gutman *et al.*, 2003;

Seymour *et al.*, 2003), which may lead to increases in defensive behavior and flight, we examined also whether administration of oCRF increases anxiety-like behavior by testing hamsters in open field (Wilson *et al.*, 1976; Crawley, 1985; Lister, 1990; Chaoulhoff *et al.*, 1994) and defensive withdrawal tests (Welker, 1959; Blanchard *et al.*, 1974; Takahashi *et al.*, 1989).

METHODS

Experiment 1

Subjects

Male Syrian hamsters were purchased from Charles River Laboratories.

Experimental animals were three to four months old and weighed 120-140 g at the start of the study. These animals were individually housed for 10 days to two weeks prior to testing in a temperature controlled ($20^{\circ}\text{C} \pm 2^{\circ}$) colony room and were maintained on a 14:10h light:dark cycle. Older animals that weighed 160-180g were housed individually and used as resident aggressors during the defeat phase of the CD protocol. Younger animals (two months) that weighed 100-110g were group-housed (five animals per cage) and were used as non-aggressive intruders during the testing phase. All animals were housed in polycarbonate cages (20 x 40 x 20 cm) with corncob and cotton bedding materials and wire mesh tops. Food and water were available ad libitum. The cages of experimental animals and resident aggressors were not changed for at least one week prior to testing to allow the animals to scent mark their territory. All training or testing occurred within the first three hours after the onset of the dark phase of the light:dark cycle in order to minimize circadian variation of the dependent measures. All experimental animals were handled daily for one week prior to training in order to habituate them to the stress of being handled by the experimenter.

Different groups of hamsters were used for each experiment. During the experiment if any animal was bitten such that it bled, the encounter was stopped and the animal was examined and treated immediately. All procedures and protocols were approved by the Georgia State University Institutional Animal Care and Use Committee.

Surgeries

Adult male Syrian hamsters were unilaterally implanted with guide cannulae aimed at the lateral ventricle (LV). Hamsters were deeply anesthetized with sodium pentobarbital (90 mg/kg) and were placed in a stereotaxic instrument. The skull was leveled between lambda and bregma prior to implantation of the guide cannula. Hamsters were then implanted unilaterally with a 4 mm, 26-gauge guide cannula aimed at the lateral ventricle. Final stereotaxic coordinates were 0.8 mm anterior and 1.1 mm lateral to bregma. The injection cannula (33-gauge) extended 1.1 mm beyond the guide to reach the final depth of -3.4 mm below dura. Dummy stylets were placed in the guide cannulae in order to keep them unobstructed. Hamsters were allowed at least one week of recovery before commencing behavioral experiments.

Experiment 1a Procedures

Experiment 1a was designed to test the hypothesis that ICV injection of ovine CRF (oCRF), a CRFR1-specific agonist, or urocortin II (UCNII), a CRFR2-specific agonist, enhances the expression of submissive behavior in previously defeated hamsters. Hamsters received either vehicle, UCNII or oCRF 30 mins prior to CD testing. The conditioned defeat model has been described elsewhere (Jasnow and Huhman, 2001) and is briefly described here. Prior to each experiment, the animals were matched by weight and randomly assigned to groups. On the training day, experimental animals were transported to a testing room.

One half of the animals underwent CD training. This training typically consists of one 15-min exposure to a resident aggressor in the aggressor's home cage. In the current experiments, however, training occurred for only 5 min in order to produce a suboptimal defeat. During each 5-min training session, the resident aggressor reliably attacked the experimental animal, and all of the experimental animals displayed high levels of submissive and defensive behavior during training. To correct for the total duration of defeat, exposure time began following the first attack by the resident aggressor (which usually occurred within the first 30 sec of the training trial). Because we have previously observed an effect of CRF on aggressive behavior in non-defeated hamsters (Faruzzi, Cooper, and Huhman, submitted), a no-defeat-vehicle group was also included for comparison. In this group, the other half of the experimental hamsters was exposed to a resident aggressor's empty cage for 5 min in lieu of being defeated, and drug was administered as described above. Twenty-four hours following defeat or novel cage exposure, experimental animals were tested for CD. The animals were transported to the testing room and a non-aggressive intruder was placed in their home cage for 5 min. Total durations of submissive, aggressive, social, and non-social behaviors were recorded during testing sessions (see *Behavioral Scoring and Analysis*). At the conclusion of testing, all hamsters were sacrificed and the brains were collected for verification of the injection site as described below.

Experiment 1b Procedures

Experiment 1b was designed to determine whether effects of oCRF on agonistic behavior observed in Experiment 1a could be due to "spillover" binding at CRFR2.

Treatment occurred as described in Experiment 1a, with the exception that hamsters received

vehicle or the CRFR2-specific antagonist ASVG in addition to oCRF. Training and testing for CD occurred as described in Experiment 1a.

Drug Preparation and Injections

UCNII (human urocortin II; Phoenix Pharmaceuticals, Belmont CA) was solubilized in 25% DMSO in sterile saline (0.9%). Hamsters received 0, 0.2 or 1.0 nmol UCNII (Valdez *et al.*, 2002). oCRF (ovine corticotropin releasing factor; Phoenix Pharmaceuticals) was solubilized in 25% DMSO in sterile saline (0.9%). Hamsters received 0, 0.10 or 1.0 nmol oCRF (Imaki *et al.*, 1996; Buwalda *et al.*, 1998; Valdez *et al.*, 2002) into the LV. ASVG (antisauvagine 30; Polypeptide Laboratories, Torrence CA) was solubilized in deionized water. Hamsters received 0 or 2.74 nmol ASVG into the LV (Takahashi *et al.*, 2001).

Microinjections were made with a 5.0 µl Hamilton syringe connected to the injection cannula via polyethylene tubing. In Experiment 1a, oCRF/UCNII/vehicle was given 30 mins prior to CD testing. In Experiment 1b, half the hamsters received ASVG/vehicle 10 mins prior to receiving oCRF/vehicle; the other half of the animals received ASVG/vehicle with oCRF/vehicle as a cocktail 30 mins prior to testing (drug solubilization was adjusted to maintain desired concentrations for either individual injections or cocktail injections). A final volume of 3.0 µl was injected over the course of 1 min for any individual injection, and the injection cannula was left in place for an additional 1 min to allow for drug diffusion. Hamsters were allowed to move freely during injections.

Following testing, each hamster was given a lethal dose of sodium pentobarbital. A volume of 0.5 µl of ink was microinjected over 30 sec using the same length injection needle as was used to administer agonists, and the brains were removed and stored in 10% formalin for at least 24h. A razor blade was used to slice through the LV. A hit was determined by

the presence of ink in the LV and a miss was assigned if the ink was primarily located above the lateral ventricles or in another location. Any animal that was assigned a miss was not used in the statistical analysis.

Behavioral Scoring and Analysis

All testing sessions were recorded and later scored by an observer blind to the experimental conditions using Noldus Observer v. 5.0 (Noldus Information Technology, Wageningen, Netherlands). During each 5 min test, the total duration (sec) of four classes of behavior were scored and grouped as follows: 1) Submissive: flee, avoid, tail up, upright and side defense, full submissive posture, stretch-attend, head flag, attempted escape from the cage; 2) Aggressive: upright and side offense, chase, and attack (including bite); 3) Social: attend, approach, investigate, sniff, nose touching, and flank marking; 4) Nonsocial: locomotion/exploration, self-groom, nesting, feeding, and sleeping. A subset of sessions was scored by a second observer, and inter-observer reliability on the total duration of submissive behavior was >90%.

The total durations of submissive, aggressive, social, and non-social behavior were individually analyzed with planned comparisons using T-tests or multivariate analysis of variance with drug and social experience as the between-subjects factors. Least Significant Difference (LSD) post hoc tests were used for pairwise comparisons. Nonparametric statistics (Mann-Whitney U tests or Kruskal-Wallis tests) were used for any comparisons with a significant Levene's test of homogeneity of variances. Alpha was ascribed at $p < 0.05$.

Experiment 2

The purpose of Experiment 2 was to determine whether the effects of oCRF on submissive or aggressive behavior are associated with increases in general anxiety-like behavior.

Subjects

Experimental hamsters were obtained and treated as described in Experiment 1, and surgeries were performed as described, as well. oCRF was prepared and administered at 0.0 or 1.0 nmol as described in Experiment 1.

Apparatus

The defensive withdrawal (DW) apparatus consisted of an open field with a withdrawal chamber inside it. The open field was a 91x91x41 cm white acrylic box, open on top, with a dark grid on the floor whose squares measured 14.5 cm² (36 squares total). The withdrawal chamber, which sat near one corner of the open field, was a black acrylic box with one side open for the animal to enter and exit. The withdrawal chamber measured 32x13x20 cm in size with an inner measurement of 30.5x13x13 cm. The field and chamber were cleaned with 100% ethanol after each animal was tested. Lighting in the field during testing was indirect and measured approximately 800-lux white light.

Procedures

Hamsters received vehicle or oCRF injection 30 min prior to testing. To begin testing, hamsters were placed inside the DW chamber and a door was closed to keep them inside it until starting the observation. The experiment began with the opening of the DW chamber, and the total duration of each test was 5 min.

Data Acquisition and Analysis

In contrast to other rodents under anxiogenic conditions (e.g., bright light vs. dim light or following social defeat), Syrian hamsters display a decreased latency to enter the open field from the DW chamber. Hamsters do exhibit rodent-typical decreases in overall locomotor activity inside an open field under anxiogenic (i.e., bright light) conditions (A.N. Faruzzi and K.L. Huhman, unpublished results). In the current experiment, the mean latency to first enter the open field from the withdrawal chamber and overall locomotor activity were compared in hamsters receiving oCRF or vehicle.

Locomotor behavior, which is a measure of anxiety as well as of sedation (Crawley, 1985; Costall *et al.*, 1989; Lister, 1990; Menard and Treit, 1999), was recorded using EthoVision Video Tracking, Motion Analysis & Behavior Recognition System, v. 3.0 (Noldus Information Technology b.v., Wageningen, The Netherlands). This system uses video input and tracks the movement of the center of gravity of the animal in the arena and stores the data on a computer where the data can be analyzed for various behaviors of interest. Total distance traveled was used to measure overall locomotor activity. Latency (in sec) to exit the withdrawal chamber was recorded from videotape with a stopwatch by an observer blind to experimental conditions. Data were analyzed using T-tests, and alpha was ascribed at $p < 0.05$.

RESULTS

Experiment 1a

Two animals were excluded from analysis due to missed cannula placement. Of the remaining animals, there was no effect of UCNII on behavior ($F(4,48)=0.53$ for submissive behavior and $F(4,48)=2.68$ for aggressive behavior, $p > 0.05$). Defeated hamsters receiving the high dose of oCRF prior to testing displayed significantly higher levels of submissive

behavior than did defeated hamsters receiving vehicle injections ($F(2,36)=7.37$, $p<0.05$). Nonsocial behavior was also significantly decreased in a dose-dependent manner ($F(2,36)=14.91$, $p<0.05$) in hamsters receiving oCRF. There were also significant increases in submissive behavior in defeated hamsters versus non-defeated hamsters receiving vehicle (i.e., defeated hamsters displayed CD) and in defeated hamsters receiving oCRF versus non-defeated hamsters receiving oCRF. Finally, non-defeated hamsters receiving oCRF displayed significantly higher levels of aggressive behavior than did defeated hamsters receiving oCRF ($F(1,17)=11.90$, $p<0.05$), and there was also a trend toward increased aggressive behavior in non-defeated hamsters receiving oCRF compared to non-defeated controls ($F(1,10)=4.07$, $p=0.07$). Means \pm SEM for each group are displayed in Figure 1.

Experiment 1b

Defeated hamsters receiving oCRF displayed significantly higher levels of submissive behavior than did controls ($F(1,21)=6.88$, $p<0.05$), and there was no significant interaction of ASVG. A trend towards higher levels of aggressive behavior was observed in all 3 drug groups (ASVG+vehicle, vehicle+oCRF, and ASVG+oCRF) compared to the vehicle only group. Additionally, there were no differences by group in animals that received ASVG/vehicle and oCRF/vehicle as a cocktail or as two separate injections. Means \pm SEM for each group are displayed in Figure 2.

Experiment 2

There was no significant difference in time to exit the withdrawal chamber in hamsters receiving oCRF compared to those receiving vehicle ($t(22)=-0.50$, $p>0.05$). Additionally, there was no significant difference in total locomotor behavior ($t(18)=0.30$, $p>0.05$).

DISCUSSION

The current data suggest that CRF modulates the expression of submissive behavior through its actions at CRFR1. ICV injection of oCRF significantly enhanced the expression of CD in defeated hamsters. Further, administration of ASVG did not block this effect, suggesting that oCRF is not working via spillover to CRFR2. The current data also show a trend for oCRF to enhance the expression of aggressive behavior in previously undefeated hamsters. In addition, the effects of oCRF appear not to be due to increases in general anxiety, as there was no effect of oCRF on DW or open field behaviors. These results suggest that activation of CRFR1 modulates agonistic behavior, in general, and that the particular behavior that is affected is determined by the animal's previous social experience.

It is difficult to make conclusions about the role of CRFR2 in agonistic behavior. The fact that UCNII had no effect on aggressive or submissive behavior suggests that CRFR2 does not play a role in the modulation of CD by CRF. However, because there is no information about binding of UCNII in hamster tissue, we cannot ignore the fact that we may have encountered a pharmacological problem and that UCNII does not adequately bind CRFR2 in hamsters. Given the fact that other research in our lab has shown effects of the CRFR2-specific antagonist ASVG on reducing CD (Jasnow *et al.*, 1999; Jasnow *et al.*, 2004b; Cooper and Huhman, 2005a), this possibility can not be dismissed. It is also possible that activation of CRFR2 is necessary for CD, but that stimulation of this receptor is not sufficient to enhance CD. Alternatively, it is possible that CRF receptors are involved in multiple aspects of the learning and display of situation-appropriate agonistic behaviors. For example, CRFR2 could be involved in memory formation of social defeat while CRFR1 could be involved modulating the actual display of appropriate behaviors given other factors

(e.g., previous social experience). Either way, further investigation is needed before conclusions can be made about the role of CRFR2 in agonistic behavior in hamsters.

Although the current results show only a trend for increased aggressive behavior in previously undefeated hamsters, we believe the data support the possibility of an excitatory effect of oCRF on aggressive behavior as we have demonstrated before (A.N. Faruzzi, M.A. Cooper, and K.L. Huhman, submitted). The effect of oCRF and oCRF plus ASVG on aggressive behavior in non-defeated hamster groups actually formed bimodal distributions. Non-defeated hamsters receiving oCRF or oCRF plus ASVG could be divided into two distinct populations, one that displayed zero seconds of aggressive behavior and 200+ seconds of submissive behavior, and another that displayed 150+ seconds of aggressive behavior and zero seconds of submissive behavior. The likely explanation for the unexpected display of submissive (and absence of aggressive) behavior in some of the “no-defeat” hamsters is that they were previously subordinate before being singly-housed for the current study. When hamsters are shipped from the breeder, they are group-housed, and they remain so after arriving at our institution until surgeries are performed. During these periods of group housing, the hamsters routinely form social hierarchies. Despite these opportunities for social experience, we have never had to prescreen hamsters for submissive/aggressive tendencies before conducting CD experiments because virtually all hamsters defeated using our typical training design subsequently show submissive behavior, regardless of their social experience prior to the experiment. It is highly likely that the no-defeat groups of hamsters that showed increased submissive behavior instead of aggressive behavior were subordinate (i.e., had been defeated) prior to this experiment. Further support for this argument is the fact that the levels of submissive behavior displayed by these hamsters were much higher than

that displayed by hamsters in the 5-min defeat groups. This suggests that these hamsters have had more substantial defeats during their period of group-housing than the 5-min defeats that were used in the current experiment, causing them to display levels of submissiveness similar to those seen in hamsters that have experienced longer defeats (Jasnow *et al.*, 2002). The addition of an experiment where hamsters are pre-screened for submissive behavior prior to assignment to a no-defeat group would offer a definitive answer to whether oCRF increases aggressive behavior in previously undefeated hamsters.

Overall, the current data support the hypothesis that activation of CRFR1 has potent enhancing effects on the expression of agonistic behavior in Syrian hamsters. Further, this effect is modulated through an unknown mechanism by the hamster's previous social experience (i.e., having been defeated or not), resulting in enhancement of either submissive behavior in previously subordinate hamsters or aggressive behavior in previously dominant hamsters, respectively.

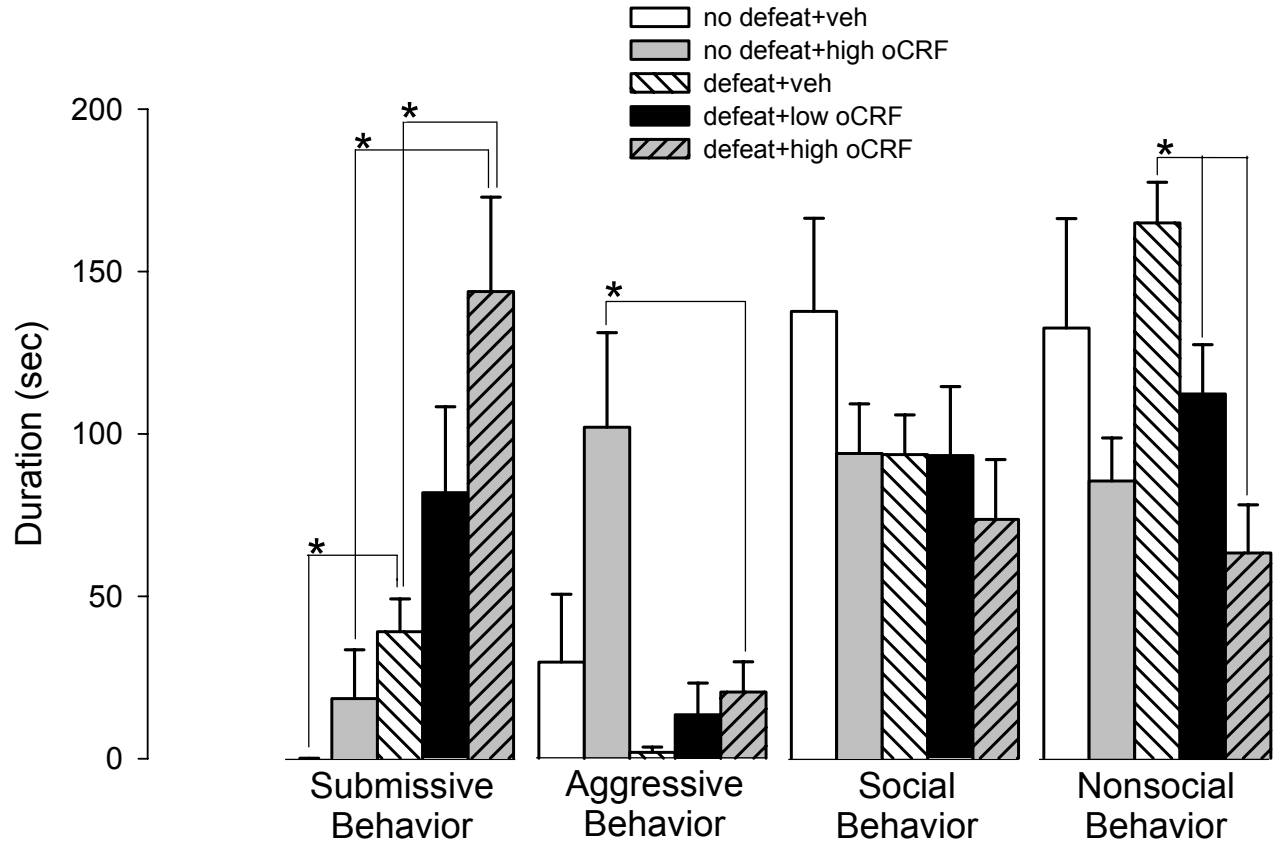
ACKNOWLEDGEMENTS

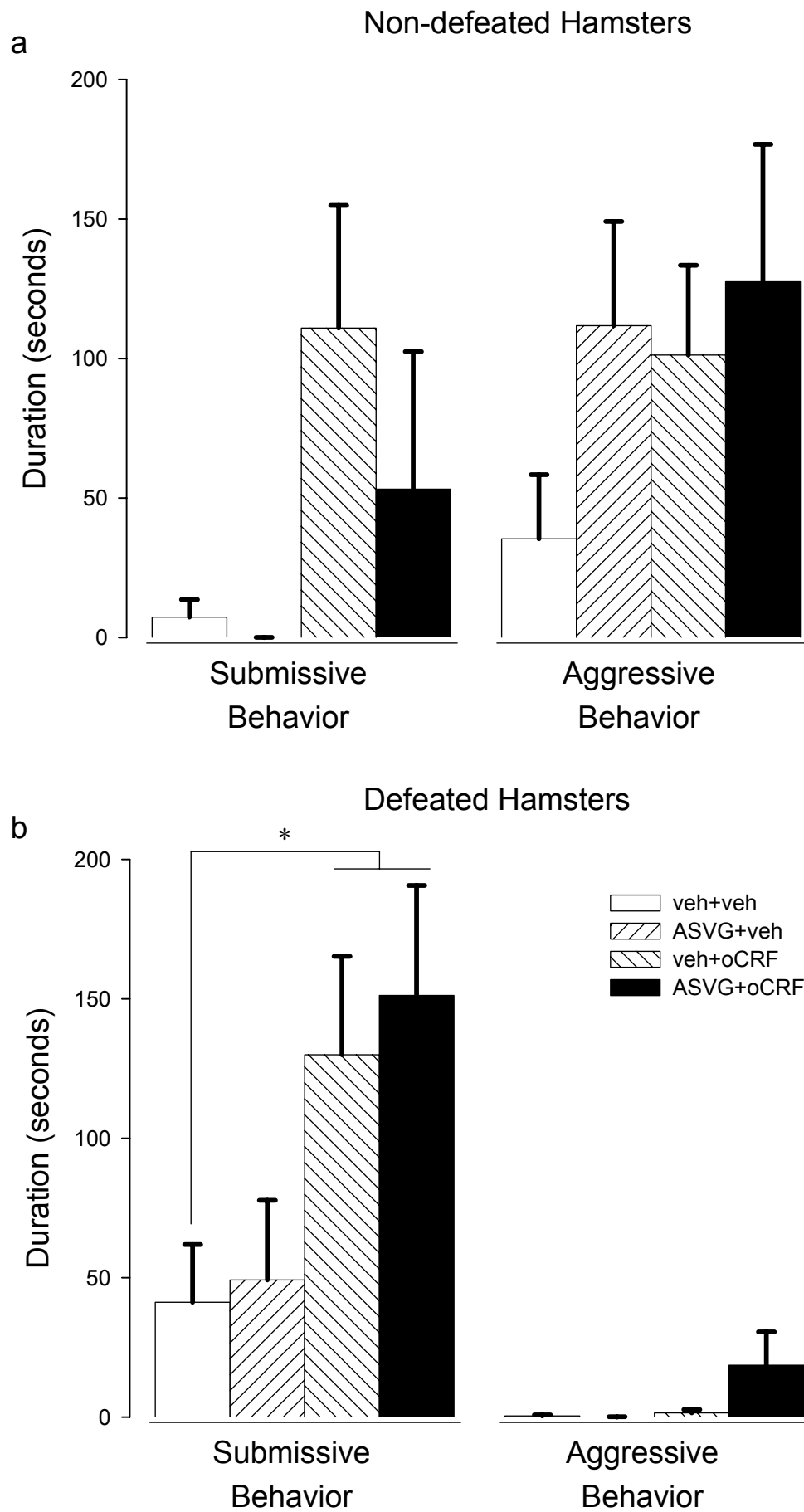
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FIGURE CAPTIONS

Figure 3.1. Means \pm SEM of duration of submissive, aggressive, social and nonsocial behavior in defeated and non-defeated male hamsters receiving oCRF or vehicle before CD testing. (planned comparisons were used to analyze this data- spurious comparisons are not illustrated; * $p < 0.05$)

Figure 3.2. Means \pm SEM of duration of submissive and aggressive behavior in a) non-defeated and b) defeated male hamsters receiving ASVG/vehicle and oCRF/vehicle before CD testing. (planned comparisons were used to analyze this data- spurious comparisons are not illustrated; * $p < 0.05$)





ABSTRACT

Corticotropin releasing factor (CRF) is located throughout the brain and has many behavioral and physiological effects. Binding of agonists to CRF receptors can cause HPA axis activation, changes in levels of anxiety or depression, decreases in feeding as well as changes in blood pressure. We have previously shown that binding of CRF to its receptors can have profound effects on agonistic behavior in Syrian hamsters. CRF receptor distribution has been studied in a number of species, but the results have been inconsistent. No reports exist of CRF receptor distribution in hamster brain. Therefore, the goal of this study was to provide an autoradiographical description of CRFR1&2 distributions throughout hamster brain. The current data reveal a number of similarities and differences with previous reports of CRFR distributions in other species. Additionally, these data provide specific information about CRFR1 and CRFR2 binding in functional subdivisions of the extended amygdala, which is of particular interest to those examining anxiety, fear responding and social conflict.

KEYWORDS: autoradiography, mapping, extended amygdala, bed nucleus of the stria terminalis, hypothalamus

INTRODUCTION

Corticotropin releasing factor (CRF) is a 41-amino acid peptide (Vale *et al.*, 1983) located in the anterior pituitary and throughout the brain (Chappell *et al.*, 1986; Eckart *et al.*, 1999; Eckart *et al.*, 2002). CRF has many behavioral and physiological effects, including increased anxiety-like responding, increased arousal and HPA axis activation, enhanced fear conditioning, and enhanced learning and memory in tasks such as visual discrimination, spatial learning, and inhibitory avoidance (Eckart *et al.*, 1999; Landgraf, 2001; Dautzenberg and Hauger, 2002; Eckart *et al.*, 2002; Grammatopoulos and Chrousos, 2002). CRF is a critical component of the HPA axis response to stress, which ultimately has a number of effects including gluconeogenesis (to sustain the “flight or flight” response) and reduced inflammation, as well as feedback to the brain to control these peripheral responses. In addition to its effects via HPA axis activation, CRF peptides and receptors are distributed throughout the brain (Bittencourt and Sawchenko, 2000; Lewis *et al.*, 2001; Reyes *et al.*, 2001; Dautzenberg and Hauger, 2002; Eckart *et al.*, 2002; Reul and Holsboer, 2002), suggesting a more direct role in behavior.

There are currently two known receptor subtypes in the CRF family (CRFR1 and CRFR2). Both subtypes are Gs protein-coupled and primarily activate adenylyl cyclase to increase cyclic AMP levels, though they are also associated with other signaling pathways (Dautzenberg and Hauger, 2002; Eckart *et al.*, 2002; Grammatopoulos and Chrousos, 2002). Binding of agonists to CRFR1 causes HPA axis activation, increased anxiety, increased depression, decreased feeding, and decreased inflammatory response. Binding of agonists to CRFR2 causes decreased feeding, decreased gastric emptying, decreased depression (except when administered into the LS), increased vasodilation, and decreased blood pressure

(Holsboer, 1999; Steckler and Holsboer, 1999; Arborelius *et al.*, 1999; Eckart *et al.*, 1999; Koob and Heinrichs, 1999; Takahashi, 2001; Dautzenberg and Hauger, 2002; Eckart *et al.*, 2002; Grammatopoulos and Chrousos, 2002). The role that CRFR2 plays in anxiety-like behavior is not well understood, as both anxiogenic and anxiolytic actions have been reported (Eckart *et al.*, 1999; Hashimoto *et al.*, 2001; Takahashi *et al.*, 2001; Dautzenberg and Hauger, 2002; Pelleymounter *et al.*, 2002; Reul and Holsboer, 2002).

There are studies mapping CRF receptor distribution in rat or rhesus monkey brain. In general, CRFR1 is found throughout the brain in areas important for sensory information processing and motor control, while CRFR2 is more restricted to subcortical regions (Reul and Holsboer, 2002). CRFR1 mRNA expression has been found in sites including the anterior pituitary, cerebral cortex, cerebellum, amygdala, hippocampal formation, hypothalamus, areas of the brain stem, septum, BST, and olfactory bulb. CRFR2 mRNA expression has been found in areas including the hippocampal formation, amygdala, LS, BST and areas of the hypothalamus and brainstem (Eckart *et al.*, 1999; Bittencourt and Sawchenko, 2000; Dautzenberg and Hauger, 2002).

Although there are numerous studies mapping CRF receptors, there is a high degree of variability in reported receptor distributions. For example, some studies report minimal CRFR1 binding or CRFR1 mRNA expression in the Ce (Radulovic *et al.*, 1999a) while others report high densities (Rybnikova *et al.*, 2003). There are also differences in reported distributions in the BST. Rominger, et al., (Rominger *et al.*, 1998) report high levels of CRFR2 binding in the BST while others report only moderate levels (Chalmers *et al.*, 1995; Radulovic *et al.*, 1999a). There are many differences other than these (for more examples see: (Chalmers *et al.*, 1995; Rominger *et al.*, 1998; Radulovic *et al.*, 1999a; Bittencourt and

Sawchenko, 2000; Rybnikova *et al.*, 2003). Many of these differences may be explained by differences in methodology. Specifically, most of these studies have examined mRNA expression while only a few have directly examined receptor densities instead of, or in addition to, mRNA expression. In one study, CRF receptor distribution in primates was examined using both autoradiography and *in situ* hybridization (ISH), and some dramatic disagreements between receptor binding and mRNA expression were found (Sanchez *et al.*, 1999). While this comparison provides some useful information about CRF receptors (i.e., receptors are likely transported to these areas from elsewhere; (Sanchez *et al.*, 1999) it highlights the problem that ensues from relying solely on mRNA expression to determine distribution of peptide binding sites.

Another possible explanation for differences among reports of CRF receptor distribution is that the neuroanatomy used for localization of receptors or mRNA is inconsistent and imprecise. There are no definitive boundaries between brain areas, and there are also numerous differences in the terminology used to delineate brain nuclei. For example, an analysis of three studies mapping CRF receptors in rat brain revealed little agreement in the neuroanatomical localization of receptors in the BST. Of the two that mapped expression in the BST, one examined only the medial division (Chalmers *et al.*, 1995), while the other examined distribution throughout the BST as divided into anteromedial, anterolateral, oval nucleus, and the posterior parts (Bittencourt and Sawchenko, 2000). In contrast, experiments investigating the role of the BST in conditioned and unconditioned fear have focused primarily on the dorsal and ventral parts of the medial and lateral divisions (Walker and Davis, 1997). Therefore, currently available data on the

distribution of CRF receptors in the BST do not provide adequate information about the localization of CRF receptors in subdivisions important for fear and anxiety-like responses.

Our laboratory studies the role of CRF receptors in agonistic behavior in Syrian hamsters (*Mesocricetus auratus*). We have shown that ICV and intra-BST injections of CRFR antagonists attenuate forms of agonistic behavior and that ICV injection of CRFR agonists enhance agonistic behavior, but we do not know where CRF acts to have these effects. We are particularly interested in CRFR distributions in the extended amygdala as well as in other areas important for emotionality, arousal, and agonistic behavior, such as the hypothalamus, PAG, and the DR. The current study provides an autoradiographical description of CRFR1&2 distributions throughout the hamster brain.

METHODS

Subjects

Male Syrian hamsters were purchased from Charles River Laboratories. Experimental animals were three to four months old and weighed 120-140 g at the start of the study. The animals were individually housed for one week prior to sacrifice in a temperature controlled ($20^{\circ}\text{C} \pm 2^{\circ}$) colony room and were maintained on a 14:10h light:dark cycle. All animals were housed in polycarbonate cages (20 x 40 x 20 cm) with corncob and cotton bedding materials and wire mesh tops. Food and water were available ad libitum, and cages were not changed while they were individually housed. Animals were handled daily during the week prior to sacrifice in order to habituate them to the stress of being handled by the experimenter. Tissue from four hamsters was ultimately used for autoradiography. All procedures and protocols were approved by the Georgia State University Institutional Animal Care and Use Committee.

CRF Receptor Autoradiography

Autoradiography procedures were performed similarly to those described previously (Sanchez *et al.*, 1999; Lim *et al.*, 2005) and are described briefly here. Receptor autoradiography was performed with [125 I-Tyr 0]-sauvagine, which has high affinity for both CRFR1 (K_d =0.2-0.4 nM) and CRFR2 (K_d =0.1-0.3 nM) (Grigoriadis *et al.*, 1996; Primus *et al.*, 1997). To identify CRFR2 binding sites, [125 I-Tyr 0]-sauvagine was combined with an excess of unlabeled CP-154,526, a CRFR1-selective antagonist (Schulz *et al.*, 1996). To identify CRFR1 binding sites, optical density readings of total CRFR binding minus specific CRFR2 were used. The subtraction technique is further described below in *Data Analysis*.

Animals were rapidly decapitated, and their brains were removed and flash-frozen on dry ice before being stored in -80°C. Brains were sliced on a cryostat at 20 μ m, and sections were thaw-mounted onto Superfrost Plus slides (Fisher, Pittsburgh, PA) and stored at -80°C until assayed. Five sets of continuous sections were collected from prefrontal cortex through hindbrain. Three adjacent sets of sections were processed for CRFR total binding, CRFR2 binding, and CRFR nonspecific binding, similar to previously described protocols (Sanchez *et al.*, 1999; Lim *et al.*, 2005).

Slides were thawed at room temperature until dry and fixed for 2 min in 0.1% paraformaldehyde-PBS solution (pH 7.4). Slides were then rinsed twice in 50 mM Tris base (pH 7.4) solution for 10 min each, then incubated in tracer for 60 min. Tracer buffer consisted of a 50 mM Tris base, 10 mM MgCl $_2$, 0.1% bovine serum albumin, 0.05% bacitracin, plus 0.2 nM [125 I-Tyr 0]-sauvagine (PerkinElmer/NEN, Boston, MA), which binds both CRFR1 and CRFR2. Slides were then rinsed with 50 mM Tris base plus 10 mM MgCl $_2$ (pH 7.4) for 3x5 min, plus 30 min with stirring on a stir plate with a magnetic bar. Slides

were then dipped in deionized H₂O, blown dry with cool air, and apposed to Kodak MR film for 72 hr with [¹²⁵I] microscale standards (PerkinElmer/NEN). Representative brain sections are shown in Figure 1.

CRFR2 Autoradiography

An adjacent set of slides were processed at the same time for CRFR2 receptor sites. CRFR2 binding was measured as described above, with the addition of unlabeled CP-154,526-1 (butyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo[2,3-d]-pyrimidin-4-yl]-ethylamine), a selective CRFR1 antagonist, which was kindly provided by Michael J. Owens, Ph.D. The concentration of CP-154,526 used in this study was 1 μM, which competes with [¹²⁵I-Tyr⁰]-sauvagine for CRFR1, but not CRFR2, binding sites because the K_i for inhibition of binding by [¹²⁵I-Tyr⁰]-sauvagine to CRFR2 is greater than 10 μM (Schulz *et al.*, 1996). This protocol has been successfully used to assay rat, monkey, and vole tissue (Sanchez *et al.*, 1999; Skelton *et al.*, 2000; Lim *et al.*, 2005). Representative brain sections are shown in Figure 1.

CRFR Nonspecific Binding

A third set of adjacent slides were processed for nonspecific binding for both CRFR1 and CRFR2 as described above in *CRF Receptor Autoradiography*, with the addition of 1 μM cold sauvagine (American Peptide, Sunnyvale, CA) to the tracer buffer containing the [¹²⁵I-Tyr⁰]-sauvagine to compete with CRFR1 and CRFR2 binding sites as described previously (Sanchez *et al.*, 1999; Lim *et al.*, 2004).

Data Analysis

The fourth and fifth sets of adjacent slides were stained for thionin and acetylcholinesterase (AChE), respectively, to better delineate brain regions (specifically

AChE for amygdalar subnuclei) for image analysis (see Figures 2 and 3). Total CRFR binding, CRFR2 binding and nonspecific binding were quantified using MCID Basic v. 7.0 (Imaging Research Inc., Ontario, Canada). AChE- and thionin-stained sections were used to delineate the borders of brain regions of interest before sampling. Bilateral measurements were averaged for each brain region across two or three sections. Optical density readings were measured in disintegrations per minute per milligram tissue (dpm/mg) based on a known set of [125 I] microsamples exposed on each film.

Specific CRFR2 binding values were obtained by subtracting background values from each section and then nonspecific binding values from [125 I-Tyr 0]-sauvagine binding in the presence of 1 μ M CP-154,526. Specific CRFR1 binding values were calculated by subtracting background values from each section and nonspecific binding, and then subtracting corrected CRFR2 binding.

Statistics

The following equations were used to obtain the corrected values for each CRFR1 and CRFR2: $(R2 - \text{background} - \text{NScor}) = R2 \text{ corrected value}$; $(R1 - R2 - \text{background} - \text{NScor} - R2 \text{ corrected value}) = R1 \text{ corrected value}$ ($\text{NScor} = \text{NS} - \text{NSbackground}$). The corrected values for each region were averaged for each animal, and an overall mean and standard deviation was calculated across animals for each brain region.

Final values for each brain region were assigned quantitative values according to respective percentile distributions of averaged, corrected values for all regions measured. Optical density was expressed as: +++, very dense binding ($\geq 65\%$); ++, dense binding ($< 65\%, \geq 45\%$); +, moderate binding ($< 45\%, \geq 25\%$); +, light binding ($< 25\%, \geq 5\%$); and +/-, sparse or no binding ($< 5\%$).

Photomicrograph Production Details

Digital images were obtained from film autoradiograms using MCID Basic v. 7.0 (Imaging Research Inc., Ontario, Canada). Digital images of AChE- or thionin-stained sections were taken using a Hewlett-Packard Scanner. Images were then imported into Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA), cropped, and minimally adjusted for brightness and contrast in order to clarify the scientific point of interest. Adobe Photoshop 7.0 was also used to add all image text and arrows.

RESULTS

Figure 1 shows CRFR distribution throughout the hamster brain. Panes labeled with “a” display CRFR1&2 distribution, and panes labeled with “b” show the corresponding CRFR2 distribution. Thionin or AChE stained sections are also displayed to aid in localization. Table 2 lists the distribution of CRFR1 and CRFR2 throughout a number of brain sites. This list highlights many areas related to emotionality and to social and agonistic behavior, which are of particular interest to our laboratory, and is not meant to be exhaustive. The following describes distributions of CRFR1 and CRFR2 in these areas.

Distribution of CRFR1

CRFR1 were distributed widely throughout the cortex (see Figure 1). Some particular prefrontal cortical areas included the Cg (Mean \pm SEM dpm/mg; Cg1=718.39 \pm 11.03, Cg2=934.83 \pm 12.48), the IL (831.01, n=1), and the PrL (1442.84, n=1). CRFR1 were also spread throughout the CPu (340.57 \pm 7.85) with a higher density in the ventral striatum (610.49 \pm 9.54). The LS and the BST had light CRFR1 binding (LS=195.30 \pm 7.64; BSTA=200.24 \pm 6.54, BSTPM=147.70 \pm 7.08, BSTPI=244.68 \pm 8.38, BSTPL=273.65 \pm 10.40). The AStr had moderate CRFR1 binding (843.94 \pm 14.84).

CRFR1 binding was spread throughout the amygdala, with the exception of the Ce, which had sparse binding (112.91 ± 6.75). The La (anterior) and MeA had moderate binding (668.35 ± 11.65 and 823.09 ± 12.31 , respectively), while the La (posterior), BLA (anterior and posterior), I, and MeP showed moderate to light binding (464.97 ± 9.69 , 344.37 ± 8.22 and 293.82 ± 8.95 , 283.06 ± 8.33 , and 276.17 ± 7.97 , respectively). However, there was dense binding in the BMA (1228.80 ± 14.92), BLV (1388.75 ± 20.75), BMP (1455.25 ± 16.62), and PMCo (1692.72 ± 12.77), and very dense binding in the PLCo (1824.26 ± 17.67).

There was sparse binding in all measured areas of the hippocampus (CA1= 46.51 ± 3.37 , CA2-3= 28.60 ± 3.38 , DG= 73.74 ± 4.10) except for the S, which had light binding (292.50 ± 5.59). The hypothalamus also showed moderate to light binding in areas including the AH (230.74 ± 5.65), LH (177.53 ± 3.76), Pa (138.34 ± 66.23), SCN (158.48 ± 5.87) and VMHL (173.94 ± 5.53), and sparse binding in the VMHM (106.21 ± 4.84) and Arc (81.58 ± 4.14).

The SuG had very dense CRFR1 binding (2660.16 ± 23.39). There was light binding in the PAG (209.47 ± 7.60) and DR (234.33 ± 10.51), and there was sparse binding in the MnR (130.10 ± 6.18) and PMnR (66.99 ± 4.64). The CG and NTS also had sparse binding (98.44 ± 5.72 and 41.13 ± 3.73 , respectively). One area that had moderate CRFR1 binding (639.98 ± 16.15) is as of yet unidentified, though when compared to the hamster atlas (Morin and Wood, 2001) it appears to overlap with areas labeled as pre-, para-, and postsubiculum (illustrated with arrow in pane 33a of Figure 1).

Distribution of CRFR2

CRFR2 were densely distributed throughout choroid plexus and therefore showed high binding in areas like the lateral ventricles (see arrow in panes 8a and 8b of Figure 1).

CRFR2 was sparsely distributed throughout the cortex or striatum (e.g., Cg1=76.73±4.27, Cg2=113.84±4.91, IL=115.85 (n=1), PrL=103.89 (n=1), CPu=89.26±5.20, and ventral striatum=99.84±5.38). There was dense binding (1959.10±20.95) in the LS. There was sparse to no binding in the BST (BSTA=137.29±5.94, BSTPI=68.62±4.49, BSTPM=2.75±1.17) except in the BSTPL, which had light binding (197.37±7.63), though binding was stronger in very discrete areas of the BSTPL (see arrow in panes 7a and 7b of Figure 1). The AStr showed moderate CRFR2 binding (971.72±10.08).

There was CRFR2 binding throughout the amygdala, with the exception of the MeA and MeP, which showed sparse binding (157.75±5.29 and 91.95±5.34, respectively). There was light to moderate binding in the Ce (276.95±2.45), BMA (302.92±5.05), La (posterior; 484.34±6.74), BLA (posterior; 514.74±5.71), BMP (526.66±8.82), BLA (anterior; 597.54±4.65) and BLV (642.62±9.48). The La (anterior) and PMCo had moderate binding (1617.92±8.61 and 1367.38±9.68, respectively), and the intercalated nucleus had dense binding (2403.82±9.95). Finally, there was very dense CRFR2 binding in the PLCo (3953.02±21.26).

As with CRFR1, there was sparse to no CRFR2 binding in the hippocampus (CA1=29.39±2.75, CA2-3=26.99±2.33, DG=31.36± 2.56, S=5.48±2.61). Much of the hypothalamus showed sparse binding in areas including the Arc (41.52±3.18), Pa (46.40±2.88), AH (149.93±5.66), and SCN (154.68±4.69). There were slightly higher levels of binding in the VMHM (206.55±4.27), VMHL (222.09±5.07), and LH (290.07±6.03).

There was sparse CRFR2 binding in the SuG (108.11±5.26). The PAG showed light binding (307.39±9.79), as did the DR (579.91±8.57), PMnR (219.09±2.55), and the NTS (which was approaching moderate; 696.99±6.75). There was sparse binding in the MnR

(155.26 ± 2.80) in the CG (29.23 ± 2.14). Finally, the unidentified area described above as overlapping with the areas labeled as pre-, para-, and postsubiculum on the hamster atlas (Morin and Wood, 2001) showed very dense CRFR2 binding (4308.08 ± 22.26 ; illustrated with arrow in pane 33b of Figure 1).

DISCUSSION

The current study is the first to illustrate the distribution of CRF receptors throughout hamster brain. The current data reveal a number of similarities and differences with previous reports of CRFR distributions in other species. Additionally, it provides specific information about CRFR1 and CRFR2 binding in functional subdivisions of the extended amygdala, which is of particular interest to those examining anxiety, fear responding and social conflict.

Although comparing our results with existing reports of CRFR distribution is difficult due to the lack of consistent techniques (as described in detail in the Introduction), it is possible to make some interesting comparisons. For example, the density of CRFR1 and CRFR2 in the LS in the current study is consistent with reports of CRFR1 and CRFR2 mRNA expression in other studies (low CRFR1 and high CRFR2; Chalmers *et al.*, 1995; Radulovic *et al.*, 1999a; Bittencourt and Sawchenko, 2000; Rybnikova *et al.*, 2003). Receptor distribution was also consistent with most reports of little to no mRNA expression in the Ce (Radulovic *et al.*, 1999a; Bittencourt and Sawchenko, 2000), with the exception of one study reporting high CRFR2 mRNA expression (Rybnikova *et al.*, 2003). On the other hand, moderate to high CRFR2 mRNA expression has been reported in the MeA (Chalmers *et al.*, 1995; Radulovic *et al.*, 1999a; Bittencourt and Sawchenko, 2000; Rybnikova *et al.*, 2003), though the present study found very minimal CRFR2 binding. Additionally, despite the differences among reports as to the division of the hippocampus, there is still a very

obvious difference in hippocampal binding in the current study overall compared to other studies. While others reported varying mRNA expression (Chalmers *et al.*, 1995; Radulovic *et al.*, 1999a; Bittencourt and Sawchenko, 2000; Rybnikova *et al.*, 2003), the current study demonstrates only sparse binding throughout most of the hamster hippocampus. Because the four other studies compared mRNA expression only, it is possible that those receptors are transported elsewhere or that post-transcriptional changes (resulting from stress, for example) are necessary for higher densities of functional CRF receptors in hamsters.

There are also some specific differences between the current and existing studies in CRFR1 and CRFR2 distribution in the hypothalamus. The current results are consistent with a previous report of little to no CRFR1 or CRFR2 mRNA expression in the Arc (Rybnikova *et al.*, 2003) but disagree with another study reporting moderate mRNA expression (Bittencourt and Sawchenko, 2000). Similarly, while other studies report CRFR2 mRNA expression to be high in the VMH (Chalmers *et al.*, 1995; Radulovic *et al.*, 1999a; Bittencourt and Sawchenko, 2000; Rybnikova *et al.*, 2003) and moderate in the Pa (Chalmers *et al.*, 1995; Radulovic *et al.*, 1999a; Bittencourt and Sawchenko, 2000), the current study reveals moderate and low CRFR2 binding in these areas, respectively.

CRF receptors have been shown to have a role in HPA axis activation, anxiety, feeding, learning and memory, conditioned defeat, and conditioned and unconditioned fear responses (Liang *et al.*, 1992; Lee and Davis, 1997; Birnbaum and Davis, 1998; Jasnow *et al.*, 1999; Eckart *et al.*, 1999; Landgraf, 2001; Faruzzi and Huhman, 2001; Dautzenberg and Hauger, 2002; Eckart *et al.*, 2002; Grammatopoulos and Chrousos, 2002; Toufexis *et al.*, 2004; Cooper and Huhman, 2005a; Cooper and Huhman, 2005b). Consistent with these findings, CRFR1 and/or CRFR2 were found in Syrian hamster brain in many regions

involved in these behaviors, such as prefrontal cortex, subdivisions of the BST and amygdala, the DR, and the LS. It has also been suggested that CRFR2 may act specifically to oppose or modulate responses mediated by CRFR1 (Reyes *et al.*, 2001; Grammatopoulos and Chrousos, 2002). The current data may provide support for this hypothesis because they reveal overlap of CRFR1 and CRFR2 in several subcortical areas.

Of particular interest, given our data on conditioned defeat, was the distribution of CRFR1 and CRFR2 in the extended amygdala. We have previously shown that non-specific and CRFR2-specific antagonists attenuate the acquisition and expression of conditioned defeat in hamsters when given into the BST (Cooper and Huhman, 2005a) but not into the Ce (Jasnow *et al.*, 2004b). It was therefore important to characterize CRFR2 distribution within subregions of the BST in order to suggest specific sites of action and to validate proposed pathways (like that described in (Jasnow *et al.*, 2004b). The current results indicate the existence of discrete densities of CRFR2 in the BSTPL that may be mediating effects of CRF antagonists on conditioned defeat. Consistent with this idea, Cooper and Huhman (Cooper and Huhman, 2005a) have demonstrated reductions in submissive behavior following injection of CRFR antagonist specifically targeted at the BSTPL. Also, because ongoing studies in our laboratory have suggested a role for the DR in the acquisition and expression of conditioned defeat (M.A. Cooper, personal communication) as well, it was of interest to characterize the distribution of CRF receptors in this region. In the current study, both CRFR1 and CRFR2 binding were observed in the DR, with a higher density of CRFR2. Finally, we have previously shown that ICV injection of a specific CRFR1 agonist increases the expression of agonistic behaviors in hamsters, suggesting a role for CRFR1 in areas important for modulating aggressive and defensive behaviors (Faruzzi, Zelinski, Tenenbaum,

and Huhman, submitted). The fact that CRFR1 binding was observed in areas such as the PAG, AH, LH, CG, and other regions related to aggression and defense systems will help pinpoint particular sites for further investigation of these effects.

Overall, CRFR1 is found throughout hamster brain while CRFR2 appears to be more confined to subcortical areas. While this finding is in agreement with most studies reporting CRF receptor distributions, there are many region-specific differences in distribution between hamster and rat and possibly differences between mRNA expression and receptor binding. The mapping presented in the current study provides a specific resource for the investigation of the role of CRF receptors in Syrian hamster behavior as well as contributes to findings obtained from investigation of other species.

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Figure 4.1. CRFR total binding (labeled with “a”) and CRFR2 binding (labeled with “b”) throughout hamster brain from prefrontal cortex to cerebellum (numbered 1-38). Pane numbers correspond to numbers on adjacent thionin-stained slides in Figure 4.2 and AChE-stained slides in Figure 4.3.

Figure 4.2. Adjacent thionin-stained slides to autoradiography slides. Numbers correspond to numbers in Figures 4.1 and 4.3.

Figure 4.3. Adjacent AChE-stained slides of the extent of the amygdala. Numbers correspond to numbers in Figures 4.1 and 4.2.

Corticotropin Releasing Factor Type 1 and 2 Receptor Distribution in Syrian Hamster Brain

Alicia N. Faruzzi^{1,3*}, Larry J. Young², and Kim L. Huhman^{1,3}

¹Center for Behavioral Neuroscience and Department of Psychology, Georgia State University, Atlanta, Georgia, 30303

²Center for Behavioral Neuroscience and Department of Psychiatry and Behavioral Sciences, Emory University, Atlanta, Georgia, 30322

³Brains & Behavior Program, Georgia State University, Atlanta, Georgia, 30303

*To whom correspondence should be addressed:

Alicia N. Faruzzi

Center for Behavioral Neuroscience

Georgia State University

P.O. Box 3966

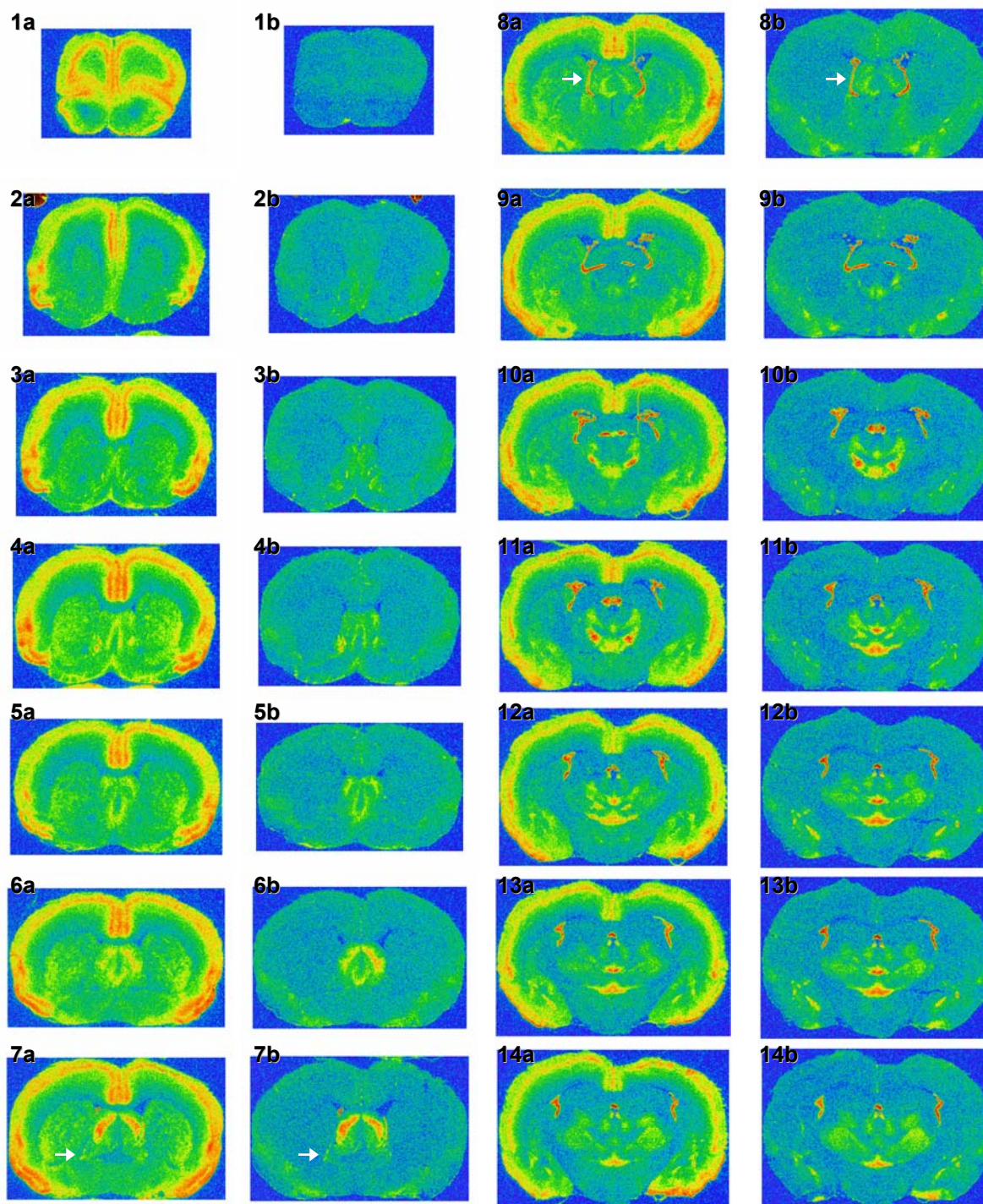
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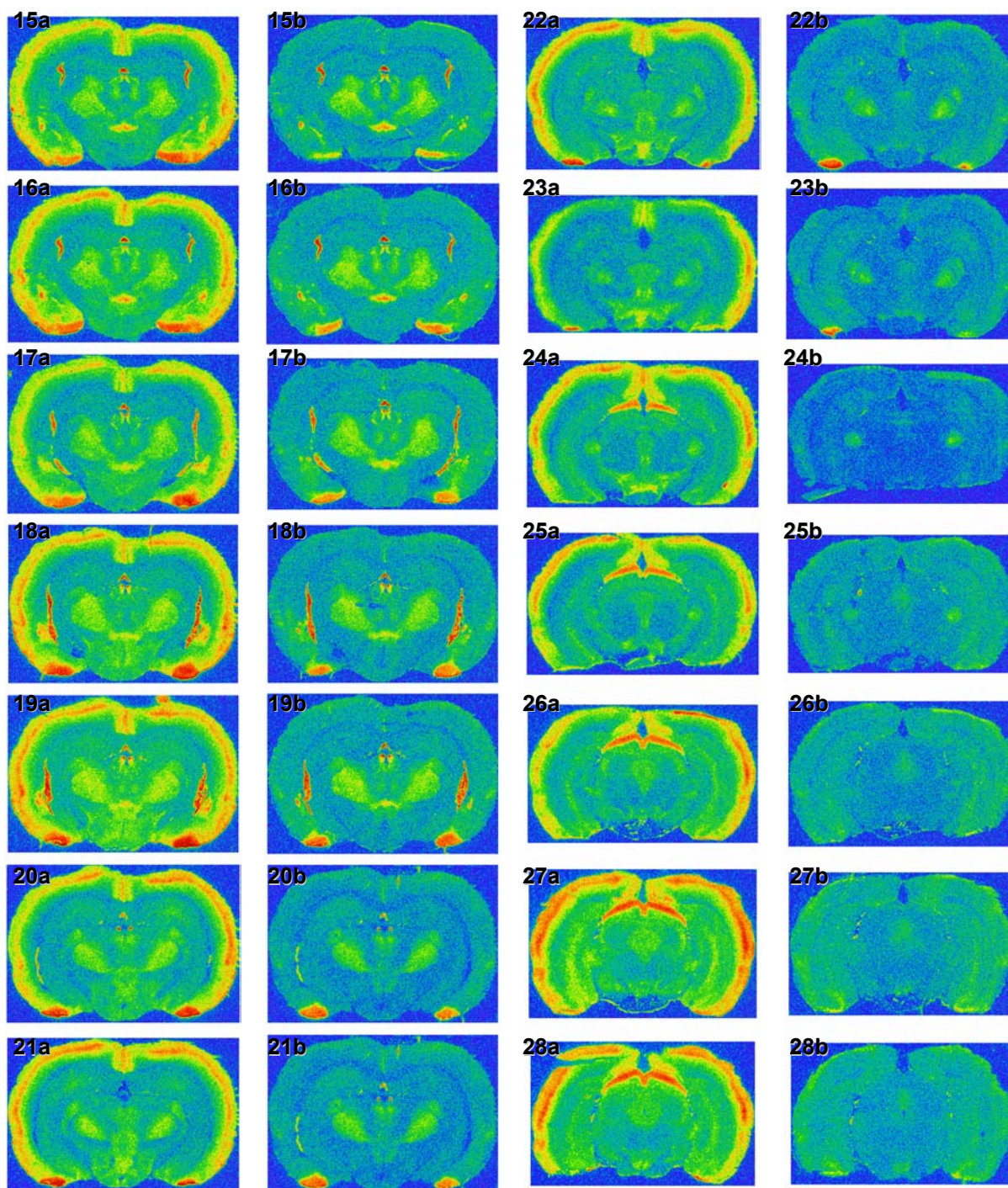
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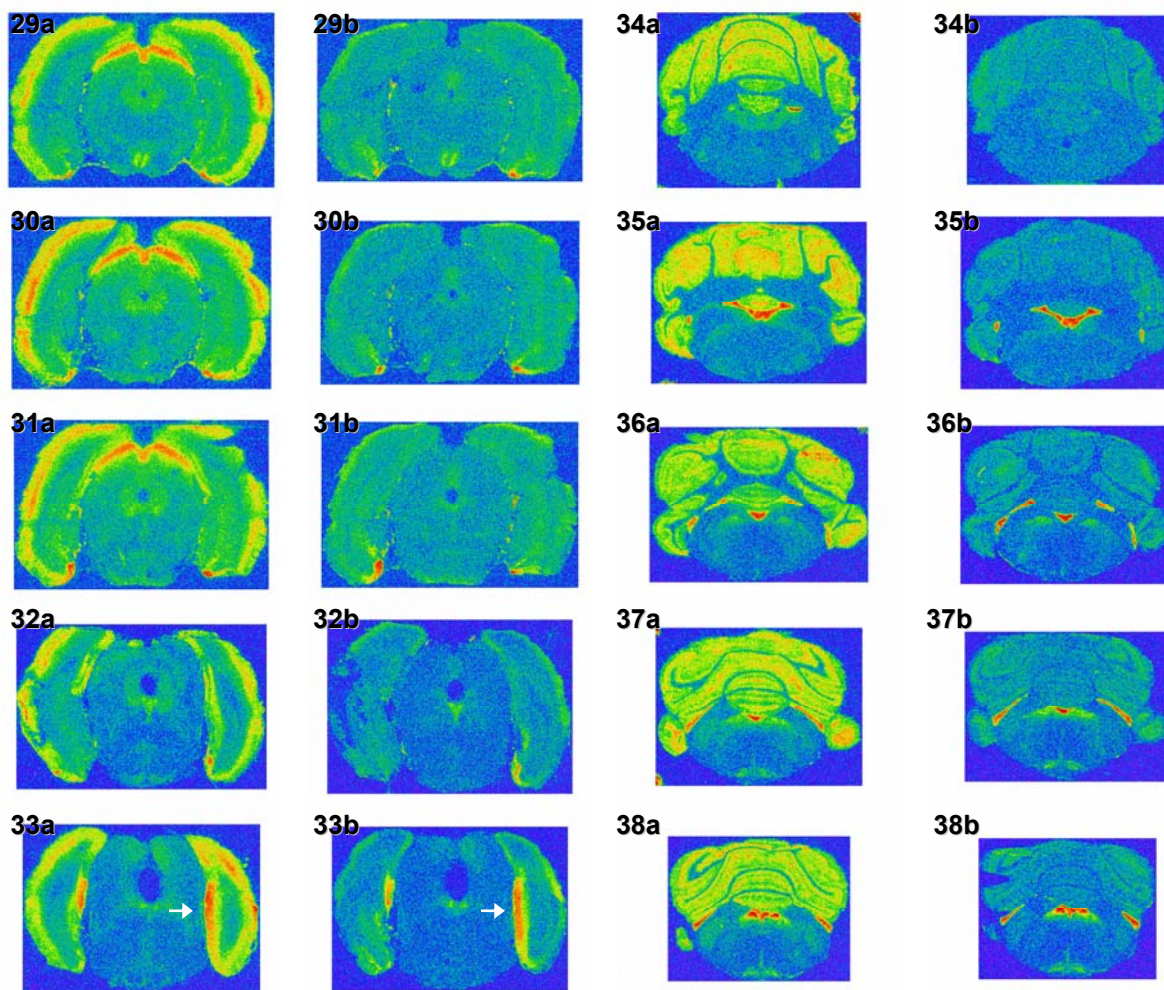
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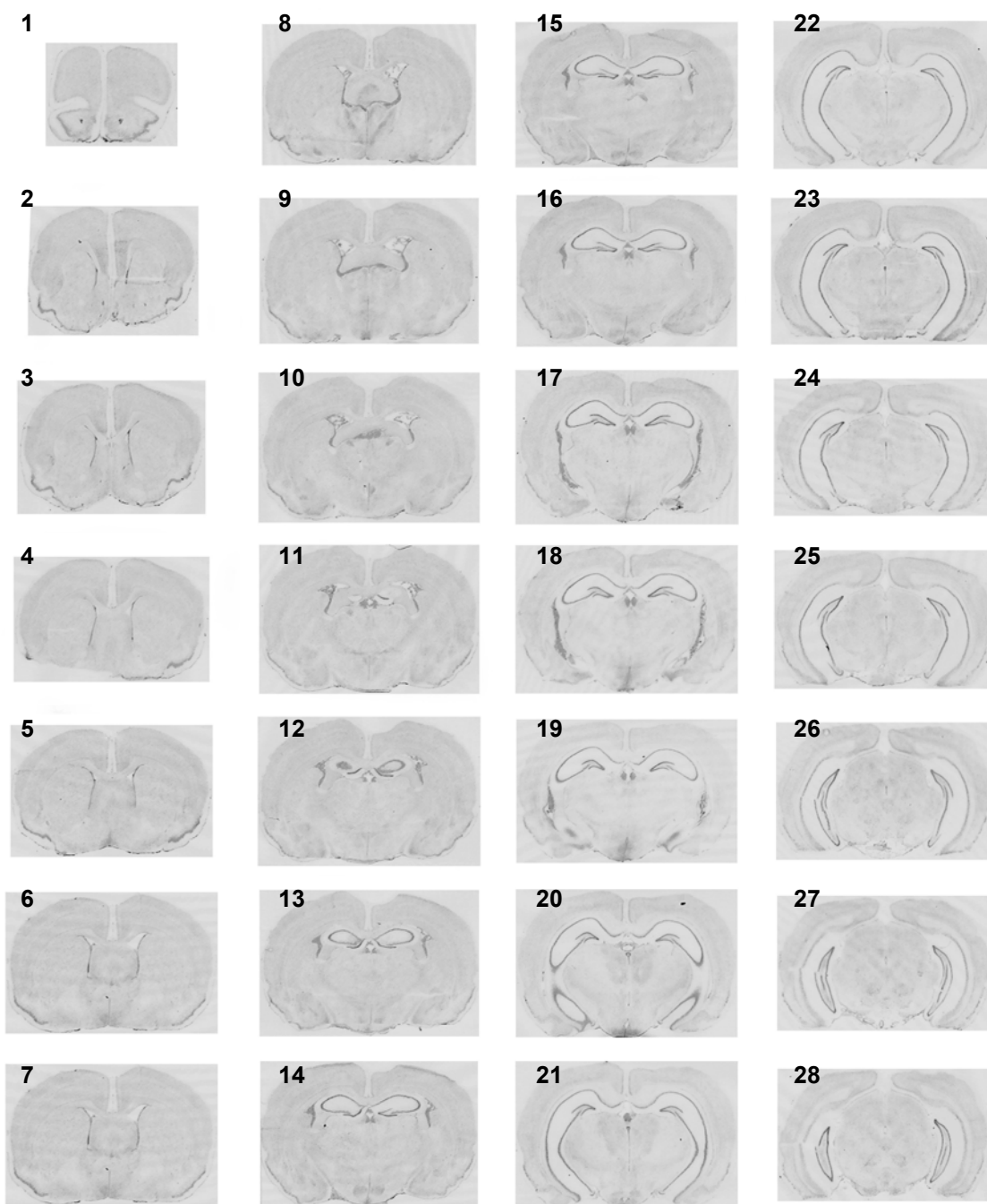
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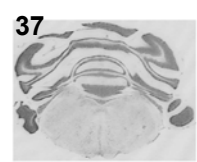
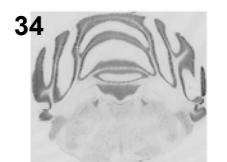
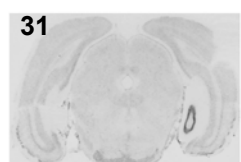
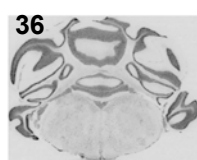
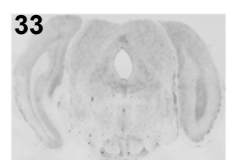
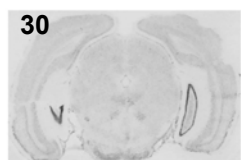
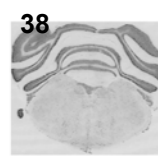
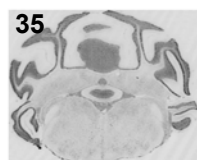
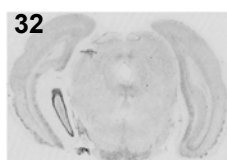
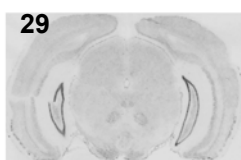
Running title: CRFR1&2 in Syrian hamster brain











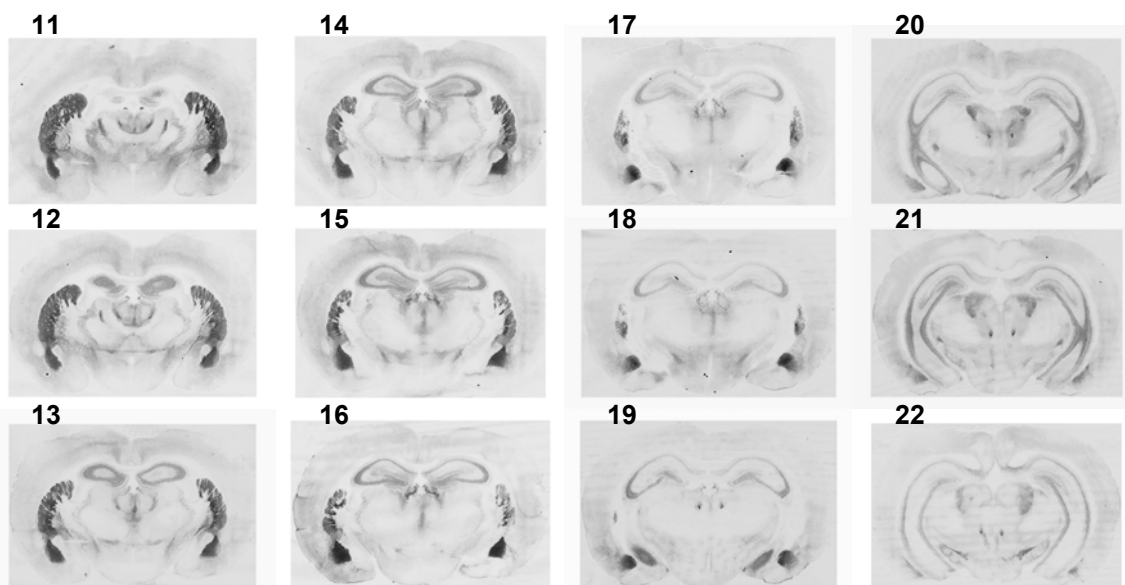


Table 4.1. Abbreviations

Region	Abbreviation
Cortex	
cingulate cortex 1	Cg1
cingulate cortex 2	Cg2
prelimbic cortex	PrL
infralimbic cortex	IL
Striatum	
caudate putamen	CPu
Lateral Septum	LS
Bed Nucleus of the Stria Terminalis	
anterior	BSTA
posteromedial	BSTPM
posterointermediate	BSTPI
posterolateral	BSTPL
Amygdalostratial Transition Area	AStr
Amygdala	
anterior lateral	La
anterior medial	MeA
intercalated	I
anterior basolateral	BLA
central	Ce
anterior basomedial	BMA
posterior lateral	La
posterior medial	MeP
ventral basolateral	BLV
posterior basomedial	BMP
posterior basolateral	BLA
posterior lateral cortical	PLCo
posterior medial cortical	PMCo
Hippocampus	
dentate gyrus	DG
subiculum	S
Hypothalamus	
anterior	AH
lateral	LH
paraventricular	Pa
suprachiasmatic	SCN
lateral ventromedial	VMHL
medial ventromedial	VMHM
arcuate	Arc
Superior Colliculus, superficial gray layer	SuG
Periaqueductal Gray	PAG
Raphe	
median raphe	MnR
paramedian raphe	PMnR
dorsal raphe	DR
Central Gray	CG
Nucleus of the Solitary Tract	Sol

Abbreviations match those used in *A stereotaxic atlas of the golden hamster brain* (Morin and Wood, 2001).

Table 4.2. ¹²⁵I-sauvagine binding to CRFR1 and CRFR2 in Syrian hamster brain

Region	CRFR1	CRFR2
Cortex		
cingulate cortex 1	++	+/-
cingulate cortex 2	++	+/-
prelimbic cortex	+++	+/-
infralimbic cortex	++	+/-
Striatum		
caudate putamen	+	+/-
ventral striatum	+	+/-
Lateral Septum	+	+++
Bed Nucleus of the Stria Terminalis		
anterior	+	+/-
posteromedial	+	+/-
posterointermediate	+	+/-
posterolateral	+	+
Amygdalostratial Transition Area	++	+
Amygdala		
anterior lateral	++	++
anterior medial	++	+/-
intercalated	+	+++
anterior basolateral	+	+
central	+/-	+
anterior basomedial	+++	+
posterior lateral	+	+
posterior medial	+	+/-
ventral basolateral	+++	+
posterior basomedial	+++	+
posterior basolateral	+	+
posterior lateral cortical	++++	++++
posterior medial cortical	+++	++
Hippocampus		
CA1	+/-	+/-
CA2-3	+/-	+/-
dentate gyrus	+/-	+/-
subiculum	+	+/-
Hypothalamus		
anterior	+	+/-
lateral	+	+
paraventricular	+	+/-
suprachiasmatic	+	+/-
lateral ventromedial	+	+
medial ventromedial	+/-	+
arcuate	+/-	+/-
Superior Colliculus, superficial gray layer	++++	+/-
Periaqueductal Gray	+	+
Raphe		
median raphe	+/-	+/-
paramedian raphe	+/-	+
dorsal raphe	+	+
Central Gray	+/-	+/-
Unidentified*	++	++++
Nucleus of the Solitary Tract	+/-	+

CRFR1 and CRFR2 distribution throughout various brain sites as determined from optical density measurements. Values are presented according to percentile distributions: ++++ very dense binding ($\geq 65\%$), +++ dense binding ($< 65\%$, $\geq 45\%$), ++ moderate binding ($< 45\%$, $\geq 25\%$), + light binding ($< 25\%$, $\geq 5\%$), +/- sparse or no binding ($< 5\%$).

*Appears to overlap with areas labeled as pre- and post-subiculum on a Syrian hamster atlas (Morin and Wood, 2001), but positive identification of this site is yet unknown.

Corticotropin Releasing Factor Receptor Binding in Syrian Hamster Brain is Not Altered by Acute Agonistic Interaction

Alicia N. Faruzzi^{1,3*}, Larry J. Young², and Kim L. Huhman^{1,3}

¹Center for Behavioral Neuroscience and Department of Psychology, Georgia State University, Atlanta, Georgia, 30303

²Center for Behavioral Neuroscience and Department of Psychiatry and Behavioral Sciences, Emory University, Atlanta, Georgia, 30322

³Brains & Behavior Program, Georgia State University, Atlanta, Georgia, 30303

*To whom correspondence should be addressed:

Alicia N. Faruzzi
Center for Behavioral Neuroscience
Georgia State University
P.O. Box 3966
Atlanta GA 30302-3966
Phone: 404-463-9440
Fax: 404-651-3929
afaruzzi98@alumni.purdue.edu

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ABSTRACT

When a male Syrian hamster is defeated in an agonistic encounter, it will subsequently fail to display territorial aggression and will, instead, display submissive behavior in the presence of a non-aggressive intruder, a response known as conditioned defeat (CD). Previous research in our laboratory has suggested roles for corticotropin releasing factor (CRF) receptor subtypes in the acquisition and expression of CD. The current study investigated whether CRF receptor binding in brain sites important for fear conditioning, expression of unconditioned fear, and defensive behavior. Tissue was collected at two time-points following defeat (24 and 72 hr), and autoradiography was performed for CRF type 1 and type 2 receptor binding. Analysis revealed no significant differences between winners, defeated hamsters, or controls at either time point in any brain area investigated. While it is acknowledged that an exhaustive examination of autoradiography results may reveal a yet-undiscovered effect, it is likely that alterations following social interaction reflect a change in peptide availability than receptor binding. Finally, directions for further investigation are discussed.

INTRODUCTION

When a male Syrian hamster (*Mesocricetus auratus*) is defeated in an agonistic encounter, it will subsequently fail to display any territorial aggression in future social encounters. A defeated hamster will instead subsequently show increased submissive behavior, even when these encounters occur in its own home cage against a smaller, non-aggressive intruder (Potegal *et al.*, 1993). This change in behavior is termed conditioned defeat (CD). In addition to this striking behavioral change, defeated hamsters exhibit increased plasma adrenocorticotrophic hormone (ACTH) and cortisol (CORT) concentrations following a social encounter (Huhman *et al.*, 1990; Huhman *et al.*, 1991; Huhman *et al.*, 1992), whereas dominant hamsters do not. These behavioral and physiological changes have led to investigation of CD as both a model of stress and of fear conditioning. These investigations have suggested, among other things, a role for corticotropin releasing factor in the expression and possibly the acquisition of CD.

Corticotropin releasing factor (CRF) is a 41-amino acid peptide (Vale *et al.*, 1983) located in the anterior pituitary and throughout the brain (Chappell *et al.*, 1986; Eckart *et al.*, 1999; Eckart *et al.*, 2002). It has many behavioral and physiological effects, including increased anxiety-like responding, increased arousal and hypothalamo-pituitary-adrenal (HPA) axis activation (Jezova *et al.*, 1999). CRF is a critical component of the HPA axis response to stress, which has a number of effects including gluconeogenesis (to sustain the “flight or flight” response), reduced inflammation, as well as negative feedback to the brain to control these peripheral responses. In addition to the critical role of CRF in the HPA axis response to stress, CRF peptides and receptors are distributed throughout the brain (Bittencourt and Sawchenko, 2000; Lewis *et al.*, 2001; Reyes *et al.*, 2001; Dautzenberg and

Hauger, 2002; Eckart *et al.*, 2002; Reul and Holsboer, 2002), suggesting a wider role for this peptide. Consistent with this view, numerous studies have demonstrated extrahypothalamic actions of CRF in various fear conditioning paradigms (Steckler and Holsboer, 1999; Radulovic *et al.*, 1999b; Takahashi, 2001) and in learning and memory tasks such as visual discrimination, spatial learning, and inhibitory avoidance (Eckart *et al.*, 1999; Landgraf, 2001; Dautzenberg and Hauger, 2002; Eckart *et al.*, 2002; Grammatopoulos and Chrousos, 2002).

Previous research in our laboratory (Jasnow *et al.*, 1999; Jasnow *et al.*, 2004b; Cooper and Huhman, 2005a) has indicated that nonselective CRF receptor (CRFR) antagonists can attenuate the expression of conditioned defeat. We have also shown that ICV administration the nonselective receptor agonist rat/human CRF enhances CD and possibly territorial aggression in hamsters that have not previously been defeated (A.N. Faruzzi, M.A. Cooper, and K.L. Huhman, submitted). In addition, we have reported that administration of the nonspecific CRF receptor antagonist D-Phe CRF (12-41) or the CRFR2-specific antagonist antisauvagine-30 attenuates the expression of CD (Jasnow *et al.*, 1999; Jasnow *et al.*, 2004b; Cooper and Huhman, 2005a), while the CRFR1 specific antagonist CP-154,526 does not (Jasnow *et al.*, 1999; Cooper and Huhman, 2005a).

After an acute defeat, the behavioral change observed in hamsters is noticeable 24 hours later and is long-lasting (Huhman *et al.*, 2003). It would therefore be reasonable to assume that there are long-term changes occurring in the brain that support the prolonged behavioral response known as conditioned defeat. It is already clear that CRF transmission plays a role in modulating the acquisition and expression of CD, so it is possible that some of these changes are occurring at the level of CRFRs. The purpose of the current experiment

was to determine whether there are differences in CRF receptor binding at several time-points post-defeat in dominant, subordinate, and isolated hamsters, and if those differences are reflective of changes in CRFR1 or CRFR2 receptor densities.

METHODS

Subjects

Male Syrian hamsters were purchased from Charles River Laboratories. Experimental animals were three to four months old and weighed 120-140 g at the start of the study. These animals were individually housed for 10 days to two weeks prior to testing in a temperature controlled ($20^{\circ}\text{C} \pm 2^{\circ}$) colony room and were maintained on a 14:10h light:dark cycle. Older animals that weighed 160-180g were housed individually and used as resident aggressors for defeats. Younger animals (two months) that weighed 100-110g were group-housed (five animals per cage) and were used as non-aggressive intruders during social interaction. All animals were housed in polycarbonate cages (20 x 40 x 20 cm) with corncob and cotton bedding materials and wire mesh tops. Food and water were available ad libitum. The cages of experimental animals and resident aggressors were not changed for at least one week prior to testing to allow the animals to scent mark their territory. All testing and tissue collection occurred within the first three hours after the onset of the dark phase of the light:dark cycle in order to minimize circadian variation of the dependent measures. All experimental animals were handled daily for one week prior to training in order to habituate them to the stress of being handled by the experimenter. Different groups of hamsters were used for each experiment. All procedures and protocols were approved by the Georgia State University Institutional Animal Care and Use Committee.

Social Interaction

Hamsters were weight-matched into three groups: winners, controls, and defeats. Social interaction for the winners group consisted of one 15-min interaction with a smaller, group-housed intruder. The resident-intruder model was chosen over a neutral arena to increase the chance of attaining each group's desired outcome, and it most closely resembles the conditioned defeat model. During the 15-min encounter, the resident experimental animal attacked the intruder and attained dominance. Social interaction for the defeat group consisted of one 15-min interaction as an intruder in the cage of a resident aggressor. A single 15-min social defeat is sufficient to produce CD (Potegal *et al.*, 1993). Treatment for control animals consisted of being placed in a resident aggressor's cage alone for 15-min, during which time the experimental hamster is exposed to the resident aggressor's bedding but is not attacked.

Tissue Collection

After behavioral training, animals were sacrificed by rapid decapitation. Weight-matched winner, control, and defeat groups were each divided into two tissue collection groups. According to group assignment, tissue was collected at either 24 or 72 hours post-training. 24-hr post-training was chosen because that is when CD is observed, suggesting that any changes in receptor densities that may occur to support CD may have occurred by this time point. 72-hr was chosen because CD has been shown to occur up to 33 days post-defeat (Huhman *et al.*, 2003), so long-term changes in receptors may not be evident until a later time point.

Following decapitation brains were extracted and flash frozen on dry ice before being stored in a -80°C freezer. Tissue was sliced on a cryostat at 20 µm. Five sets of sequential

sections targeted at specific points in the brain were collected and mounted on Fisher Superfrost slides and stored in a -80°C freezer until autoradiography was performed. Two adjacent sets of sections were processed each for CRFR total binding or CRFR2 binding, similar to previously described protocols (Sanchez *et al.*, 1999; Lim *et al.*, 2005), and remaining sets were stained or reserved.

CRF Receptor Autoradiography

Autoradiography procedures were performed similarly to those described previously (Sanchez *et al.*, 1999; Lim *et al.*, 2005) and are described briefly here. Receptor autoradiography was performed with [125 I-Tyr 0]-sauvagine, which has high affinity for both CRFR1 (K_d=0.2-0.4 nM) and CRFR2 (K_d=0.1-0.3 nM) (Grigoriadis *et al.*, 1996; Primus *et al.*, 1997). To identify CRFR2 binding sites, [125 I-Tyr 0]-sauvagine was combined with an excess of unlabeled CP-154,526, a CRFR1-selective antagonist (Schulz *et al.*, 1996). To identify CRFR1 binding sites, optical density readings of total CRFR binding minus specific CRFR2 were used. The subtraction technique is further described below in *Data Analysis*.

Slides were thawed at room temperature until dry and lightly fixed for 2 min in 0.1% paraformaldehyde-PBS solution (pH 7.4). Slides were rinsed twice in 50 mM Tris base (pH 7.4) solution for 10 min each, and then incubated in tracer for 60 min. Tracer buffer consisted of a 50 mM Tris base, 10 mM MgCl₂, 0.1% bovine serum albumin, 0.05% bacitracin, plus 0.2 nM [125 I-Tyr 0]-sauvagine (PerkinElmer/NEN, Boston, MA), which binds both CRFR1 and CRFR2. Following incubation, slides were rinsed with 50 mM Tris base plus 10 mM MgCl₂ (pH 7.4) for 3x5 min, plus 30 min with stirring on a stir plate with a magnetic bar. Slides were then dipped in deionized H₂O, blown dry with cool air, and apposed to Kodak MR film for 72 hr with [125 I] microscale standards (PerkinElmer/NEN).

CRFR2 Autoradiography

An adjacent set of slides were processed at the same time for CRFR2 receptor sites. CRFR2 binding was measured as described above, with the addition of unlabeled CP-154,526-1 (butyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo[2,3-d]-pyrimidin-4-yl]-ethylamine), a selective CRFR1 antagonist, which was kindly provided by Michael J. Owens, Ph.D. The concentration of CP-154,526 used in this study was 1 μ M, which competes with [125 I-Tyr⁰]-sauvagine for CRFR1, but not CRFR2, binding sites because the K_i for inhibition of binding by [125 I-Tyr⁰]-sauvagine to CRFR2 is greater than 10 μ M (Schulz *et al.*, 1996). This protocol has been successfully used to assay rat, hamster, monkey, and vole tissue (Sanchez *et al.*, 1999; Skelton *et al.*, 2000; Lim *et al.*, 2005).

Data Analysis

Third and fourth sets of adjacent slides were stained for AChE and thionin to better delineate brain regions (specifically AChE for amygdalar subnuclei) for image analysis. Total CRFR binding, CRFR2 binding and nonspecific binding were quantified using MCID Basic v. 7.0 (Imaging Research Inc., Ontario, Canada). AChE- and thionin-stained sections were used to delineate the borders of brain regions of interest before sampling. Measurements were averaged for each brain region across two or three sections. Optical density readings were measured in decompositions per minute per milligram tissue (dpm/mg) based on a known set of [125 I] microscales exposed on each film.

Specific CRFR2 binding values were obtained by subtracting background values from each section and then nonspecific binding values from [125 I-Tyr⁰]-sauvagine binding in the presence of 1 μ M CP-154,526. Specific CRFR1 binding values were calculated by

subtracting background values from each section and nonspecific binding, and then subtracting corrected CRFR2 binding

Statistics

The following equations were used to obtain the corrected values for each CRFR1 and CRFR2: $(R2 - \text{background}) = R2 \text{ corrected value}$; $(R1R2 - \text{background} - R2 \text{ corrected value}) = R1 \text{ corrected value}$. The corrected values for each region were averaged for each animal, and an overall mean and standard error was calculated across animals in each group for each brain region. Means were then compared using a one way analysis of variance. Alpha was set at 0.05.

RESULTS

The following regions were quantified: lateral septum (LS), posterolateral bed nucleus of the stria terminalis (BSTPL), lateral amygdala (La), and piriform cortex (Pir). Of these regions (LS, BSTPL, La, Pir), no significant differences in binding were found (CRFR1: $F(5,47)=0.76$, $F(5,22)=0.58$, $F(5,23)=0.22$, $F(5,23)=0.88$, respectively; CRFR2: $F(5,48)=1.09$, $F(5,23)=1.42$, $F(5,23)=0.51$, $F(5,23)=1.04$, respectively; $p>0.05$). Non-statistical analysis of other brain sites (including periaqueductal gray (PAG) and dorsal raphe (DR)) revealed no differences in CRFR1 or CRFR2 binding, and no more measurements were recorded.

DISCUSSION

The current study revealed no differences in CRFR1 or CRFR2 binding between winners, controls, and defeated hamsters in a number of sites analyzed. The most parsimonious interpretation of these data is that there are no changes in CRFR1&2 binding following acute defeat. There are many references to CRF or CRF-like peptide changes

following various stressors (Chappell *et al.*, 1986; Watts, 1996; Hatalski *et al.*, 1998; Hsu *et al.*, 1998; Helmreich *et al.*, 1999; Jezova *et al.*, 1999; Hsu *et al.*, 2001). On the contrary, there are fewer examples of changes in receptor binding (see Fuchs and Flugge, 1995). Although it may be that little research has been dedicated to examining binding sites for CRF-like peptides following stress, it is possible that this bias in the literature suggests that changes in peptide availability following stress are more likely to occur than are changes at the level of CRF receptors.

It is also possible, of course, that CRF receptors are altered by defeat and that the current design did not reveal this change. While it is possible that there were problems at the level of the assay performed, it is more likely that the behavioral design was not conducive to detecting changes. Instead of collecting tissue at time points following the initial defeat, the tissue could have been collected at various time points following a 5-min CD test (exposure of a defeated hamster to a non-aggressive intruder). If CRF peptide availability increases in defeated hamsters, increased CRF peptide release during CD testing might result in *decreased* binding of ^{125}I -sauvagine (during autoradiography) compared to controls because those binding sites are already bound by endogenous CRF.

Although it is possible that an exhaustive analysis of the autoradiography data might reveal an effect that has not been observed thus far, analysis was focused on limbic sites previously associated with defeat, particularly the BSTPL (Jasnow *et al.*, 2004b; Cooper and Huhman, 2005a), and with fear conditioning (LeDoux, 2000; Blair *et al.*, 2001; Repa *et al.*, 2001), as well as the LS, DR, and PAG, which is associated with defensive behavior. The LS is involved in the control of agonistic behavior in hamsters (Kollack-Walker *et al.*, 1997; Delville *et al.*, 2000) and in fear conditioning (Campeau *et al.*, 1997; Vouimba *et al.*, 1998;

Desmedt *et al.*, 1999; Vouimba *et al.*, 1999; Radulovic *et al.*, 2000). The DR is also involved in fear circuitry and is a known site for CRF modulation of serotonin (Price *et al.*, 1998; Kirby *et al.*, 2000; Hammack *et al.*, 2002; Thomas *et al.*, 2003; Waselus *et al.*, 2005). Finally, the PAG is important for fearful/defensive responses (Misslin, 2003; Vianna and Brandao, 2003; Vianna *et al.*, 2003). It was expected that any differences in binding between groups would be evident in one of these sites hypothesized to be important in CD and dominance/subordinance formation. It is possible that changes may have occurred in a site we did not anticipate and would only be discovered during an analysis of numerous brain sites. Though this may be performed at a later date, an analysis of the data at that level is beyond the scope of this study.

It is more likely, however, that a change in peptide availability and not a change in available binding sites accounts for defeat-induced behavioral changes mediated by CRF. This conclusion is drawn from the fact that exogenous CRF administration enhances submissive behavior in suboptimally defeated hamsters. These hamsters, when given vehicle, do not show CD, suggesting that changes did not occur following the previous defeat that could support an alteration in subsequent behavior. However, central administration of ovine CRF to these hamsters before exposure to a non-aggressive intruder has dramatic effects on subsequent behavior (i.e., they exhibit high levels of CD). Therefore, it appears that changes in binding availability may not be occurring following defeat and that increases in submissiveness are only observed if the quantity of available peptide increases.

Overall, this experiment does not reflect a dead-end but a point at which we can reconsider potential mechanisms and ways to investigate them. Hopefully, future

experiments will provide more information toward understanding the role of CRF receptors in agonistic behavior.

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GENERAL DISCUSSION

Synthesis of the Dissertation

The goal of this project was to investigate the role of CRF type 1 and 2 receptors in CD and to identify neuroanatomical locations where CRF may be acting. The first study showed that exogenous administration of a non-specific CRF agonist enhances the expression of submissive, and possibly aggressive, behavior in hamsters that have previous social experience. Submissive behavior is increased in hamsters receiving suboptimal (less than the standard 15-min) defeat training sessions, while aggressive behavior appears to be increased in hamsters not previously subjected to defeat during the experiment. The second study aimed to differentiate which receptor subtype, CRFR1 or CRFR2, mediates effects of r/hCRF on agonistic behavior by giving CRFR subtype-specific agonists before testing. This study also aimed to further examine effects of CRF on aggressive behavior with additional control. Further, experiments were performed to determine whether observed effects on submissive and aggressive behavior were specific to agonistic behavior or if they resulted from general increases in anxiety-like behavior. The results of this study strongly suggest that CRFR1 mediates effects of CRF on agonistic behavior, that the effects are modulated by previous social experience, and that the effects do not appear due to increased anxiety-like behavior. The third and fourth studies aimed to describe the localization of CRFR subtypes throughout hamster brain and to determine whether there are social experience-induced changes in receptor binding of a radiolabeled ligand (possibly indicating increases in binding availability for endogenous CRF ligands). While we successfully mapped CRFR subtypes throughout the hamster brain, thereby identifying potential areas of CRFR1 activity related to agonistic behavior and CD, we did not see changes in receptor binding

following winning or losing an acute agonistic encounter compared to controls. Though other possibilities for this lack of change are also proposed in study four, it is suggested that any changes in CRF neurotransmission are not at the level of the receptor but at the level of the peptide. This possibility is supported by numerous reviews of stress-induced changes in CRF peptide or mRNA expression (Chappell *et al.*, 1986; Watts, 1996; Hatalski *et al.*, 1998; Hsu *et al.*, 1998; Helmreich *et al.*, 1999; Jezova *et al.*, 1999; Hsu *et al.*, 2001).

The most important relationship highlighted by these results is that CRFR1 activity enhances the expression of agonistic behavior and that this increase appears to be modulated by the animal's previous social experience. In contrast to this finding, other research in our laboratory has revealed a relationship between CRFR2 and submissive behavior.

Administration of non-specific or CRFR2-specific antagonists, either ICV or directly into the BST, attenuate the acquisition and expression of CD while administration of a CRFR1-specific antagonist does not (Jasnow *et al.*, 1999; Jasnow *et al.*, 2004b; Cooper and Huhman, 2005a; Cooper and Huhman, 2005b). This seeming contradiction may indeed indicate that CRFR1 and CRFR2 are involved in different aspects of the learning and the display of situation-appropriate agonistic behaviors. Because CRFR2 antagonists reduce the acquisition of CD while neither CRFR1 agonists or antagonists (Jasnow *et al.*, 1999; Cooper and Huhman, 2005b) affect acquisition, CRFR2 could be involved in memory formation and retrieval of social defeat. CRFR1 could then be involved in the expression of specific agonistic behaviors after learning and retrieval have occurred.

The idea that CRFR1 is involved in the output of behavioral responses predetermined by previous social experience raises questions about where and how CRFR1 is affecting the expression of agonistic behavior. While it is not possible with the current data to outline

specific pathways and mechanisms involved, examining the distribution of CRFR1 in the context of the neural control of agonistic behavior suggests possible directions for further investigation. The first question to address is at what point in the expression of agonistic behavior CRFR1 might be playing a role. Siegel et al. (1999) proposed two classes of pathways involved in the “attack response” that may be applicable to this discussion. The first pathway is involved in “mediating expression,” that is, acting at the level of neural signals necessary for carrying out motor and autonomic responses. The second pathway is involved in “modulating expression,” or modulating a “threshold” for eliciting the expression of agonistic behavior (i.e., suppression or facilitation of behavioral output). If CRFR1 are involved at the level of “mediating expression,” then blocking CRFR1 should block the expression of agonistic behavior. However, this role is not likely because administration of the CRFR1-specific antagonist CP-154,526 does not affect CD (Jasnow *et al.*, 1999; Cooper and Huhman, 2005b). A more likely role for CRFR1 lies at the level of “modulating expression” of agonistic behavior by affecting “thresholds,” via some unknown pathway, necessary for the display of agonistic behavior. Because oCRF (a CRFR1-specific agonist) increases agonistic behavior in suboptimally defeated hamsters, it is possible that the concomitant increase of CRFR1 activity is occurring at the level of this/these unspecified “threshold(s).” By doing so, oCRF may be facilitating the attainment of a necessary threshold for initiating the expression of agonistic behavior. On the other hand, CRFR1 antagonists have no effect on the expression of submissive behavior in optimally (15-min) defeated hamsters. It is possible that these hamsters have undergone changes at this “threshold” level or at a level downstream of this point such that CRFR1 activity is no longer necessary to elicit the expression of agonistic behavior.

Although it is not clear where CRFR1 activation is occurring to affect agonistic behavior, the idea that CRFR1 is modulating the expression of agonistic behavior (as discussed above) helps to narrow the list of potential sites of action. According to Siegel *et al.* (1999), the location where “modulation of expression” occurs is likely to be limbic. Of the limbic sites thought to be involved in aggressive and defensive behavior in hamsters, the LS shares reciprocal connections with the AH (the latter is considered the center of the neural network controlling offensive aggression in hamsters and in other species; Albert and Walsh, 1984; Siegel *et al.*, 1999; Delville *et al.*, 2000) and shows increases in *c-fos* mRNA following acute defeat (Kollack-Walker and Newman, 1995; Kollack-Walker *et al.*, 1997).

Additionally, CRFR1 binding was observed in the LS in the current study, confirming the existence of CRFR1 in this structure. Thus, the LS is a possible site of action for CRFR1 binding in enhancing agonistic behavior. Perhaps the LS is exerting inhibitory control on aggressive behavior that is enhanced by CRFR1 binding in previously defeated hamsters, thereby facilitating the suppression of aggressive behaviors to allow for enhanced expression of defensive behavior. On the other hand, CRFR1 binding may be lower in hamsters that have not been defeated, thereby facilitating the expression of aggressive behavior.

Another limbic site involved in both aggressive and defensive behaviors is the BST. Acutely defeated hamsters express increases in *c-fos* mRNA in the BST (Kollack-Walker and Newman, 1995; Kollack-Walker *et al.*, 1997), which, like the LS, shares reciprocal connections with the AH (Delville *et al.*, 2000). The BST also expresses *c-fos* immunolabeling following a display of offensive aggression (specifically in the posteromedial division of the BST; Delville *et al.*, 2000). Because CRFR1 are expressed throughout the BST in hamster, the BST may be another possible site of CRFR1 activity

involved in enhancing agonistic behavior. Presently, it is difficult to propose a mechanism for these actions. However, the reciprocal connections between the BST and AH enter the posterior BST, and *c-fos* immunolabeling after offensive aggression occurs in the BSTPM (Delville *et al.*, 2000). Additionally, increases in *c-fos* mRNA expression following acute defeat occur in the BSTA. It is therefore possible that the BSTPM and BSTA are potential sites of CRFR1 binding for enhancing aggressive or defensive behavior, respectively.

It is likely that there are other possible sites of action, or different sites for particular aggressive and defensive behaviors, where CRFR1 activity can alter agonistic behavior in a modulatory fashion. However, these hypotheses allow for continued investigation of this phenomenon in an effort to further examine the role of CRFR1 in the enhancement of agonistic behavior.

Future Directions

The LS is the most straightforward place to begin an investigation of other limbic sites that may mediate the effects of CRFR1 activation on agonistic behavior. Similar to the experimental design used in the agonist studies in the current project, hamsters could be implanted with a guide cannula into the LV and the LS. By injecting a CRFR1 antagonist (like CP-154,526) into the LS prior to ICV injection of oCRF, CRFR1 in the LS should be effectively blocked by the antagonist, preventing binding of oCRF. If the LS is the site where CRFR1 activation is occurring to enhance agonistic behavior, enhancement should not occur in hamsters receiving CP-154,526. Figure 1 of the Appendix proposes potential pathways that originate in the LS.

Assuming the LS is the site of CRFR1 activation, its reciprocal connections with the AH would provide a pathway for the enhancement of aggressive behavior. It is possible that

inhibitory control of aggressive behavior from the LS to the AH may be “turned off,” or inhibited, by CRFR1 activation in the LS in animals that have not been previously defeated. In this way, aggressive behavior is not being suppressed (and may actually be increased). Conversely, CRFR1 activation in a previously defeated hamster may facilitate inhibition of aggression through LS→AH connections, decreasing the likelihood of displaying aggressive behavior. Assuming the enhancing effects of CRFR1 activity on aggressive and submissive behavior are occurring via the same site of action, it is possible that connections from the LS to the AH also activate mechanisms that initiate defensive behavior. This idea is supported by the fact that *c-fos* mRNA increases in the AH in hamsters following acute defeat (Kollack-Walker and Newman, 1995; Kollack-Walker *et al.*, 1997). Alternatively, another possible pathway for activating defensive behavior could be through LS→ extended amygdala connections. In particular, the Ce or BSTL, both of which are involved in initiation of autonomic and endocrine responses to stress, may be important for activating defensive behavior. As it is difficult to differentiate brain regions involved in stress responding from specific regions involved only in defensive behavior (Kollack-Walker and Newman, 1995; Kollack-Walker *et al.*, 1997), it is likely that defensive behavior in submissive hamsters is modulated by the same upstream sites that are stimulating the accompanying stress response. Potential candidates include Ce, BSTPL, IL, LS, and DR. These sites provide potential targets to further map out the circuitry controlling the effect of CRFR1 activation on agonistic behavior. Finally, once it is determined to where the LS is projecting to affect agonistic behavior, it will be necessary to determine, on a molecular level, how CRFR1 binding is activating these pathways and how previous social experience is modulating its effects.

A similar experimental design to that described above could be applied to investigation of the BST in mediating the effects of CRFR1 activation on agonistic behavior. However, this approach is complicated by the fact that CRFR1 activity could be occurring in discreet, and possibly multiple, subdivisions of the BST to enhance the experience-appropriate behavior. Connections between the BSTP and AH could mediate enhancement of aggressive behavior in hamsters that have not been previously defeated. If so, the enhancement of aggressive behavior may be blocked by CP-154,526 injection into the BSTPM prior to ICV injection of oCRF. Investigating the site of action of CRFR1 activation on defensive (or submissive) behavior is more complicated. Increased *c-fos* mRNA expression has been observed in the BSTA following defeat, making this site a possible candidate for oCRF binding. However, because defensive behavior and stress response circuitry are likely overlapping, the BSTPL may be a possible site of action due to its connections to the stress system (Walker and Davis, 1997; Davis *et al.*, 1997; Davis and Shi, 1999; Lang *et al.*, 2000). Therefore, both the BSTPL and the BSTA (specific anterior subdivision unknown) are candidates for receiving CP-154,526 prior to ICV oCRF. Figure 2 of the Appendix proposes potential pathways that originate in the BST. If antagonism of CRFR1 in one of these sites blocks enhancement of submissive behavior by oCRF in previously defeated hamsters, further investigation can commence as to the specific molecular mechanisms involved in this effect and how previous social experience modulates this outcome. In the event that antagonism of CRFR1 in both BSTA and BSTPL block enhancement of submissive behavior, it is possible that there are connections from or between these two sites that are involved in the control of this effect.

As stated previously, these are only starting points for further investigation. Despite the numerous findings of this project, an understanding of CRF enhancement of agonistic behavior is far from being attained. I have essentially come a long way to know virtually nothing about this phenomenon, but I look forward to the challenge of one day answering these questions.

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APPENDIX

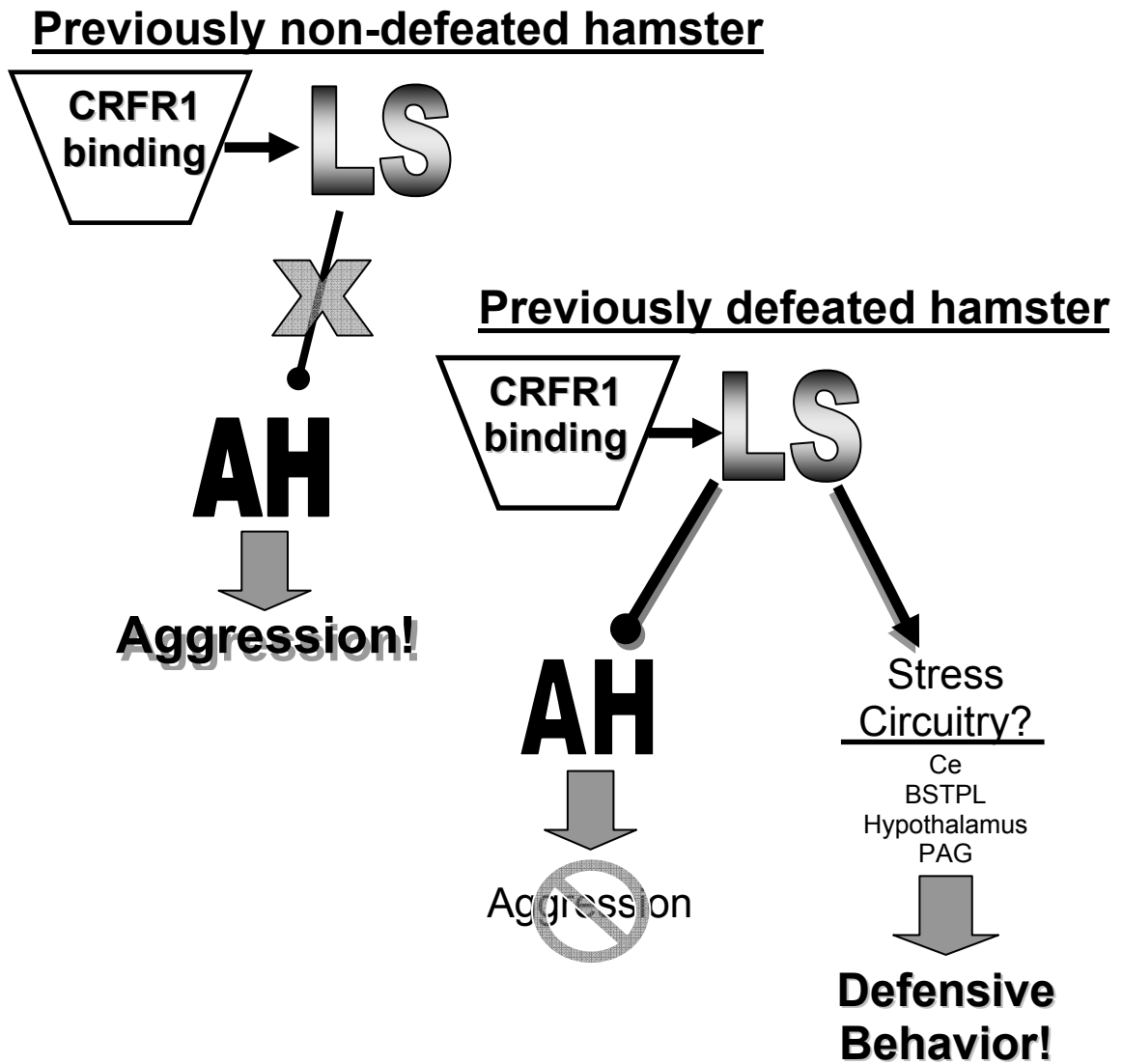


Figure A.1. Represents potential circuitry involved in the control of agonistic behavior by CRFR1 in the LS. In non-defeated hamsters, CRFR1 activity blocks the inhibitory control of the LS over the AH, which allows for the expression of aggressive behavior. Conversely, in previously defeated hamsters, CRFR1 in the LS results in inhibition of aggressive behavior by inhibiting the AH. Additionally, projections from the LS to stress circuitry (such as the extended amygdala and other sites) would likely mediate the effects of CRFR1 on defensive behavior (these projections may be direct or indirect).

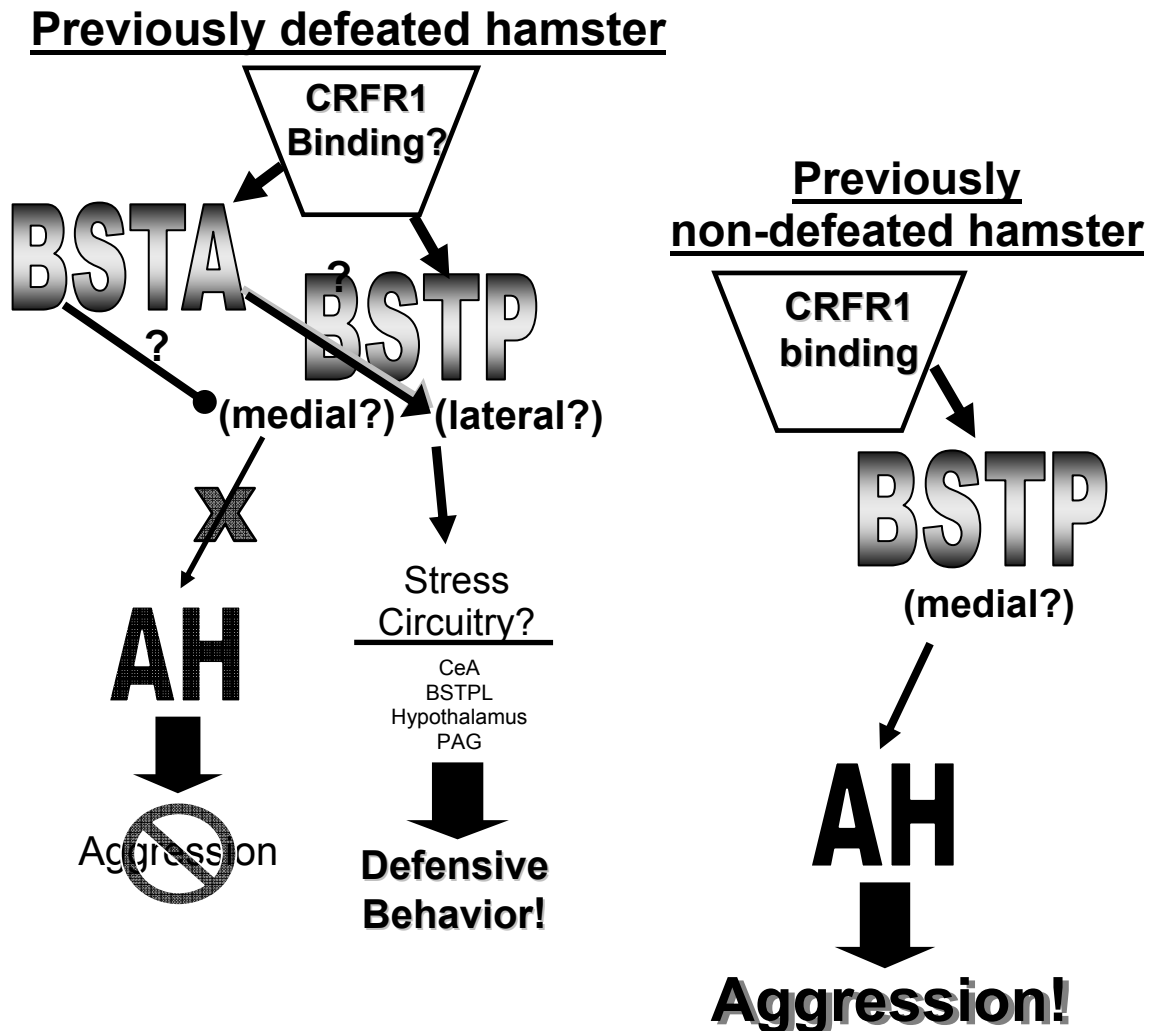


Figure A.2. Represents potential circuitry involved in the control of agonistic behavior by CRFR1 in the BST. In previously defeated hamsters, CRFR1 activation could be occurring in the BSTPL, initiating stress-responsive behavior (including defensive behavior). In non-defeated hamsters, CRFR1 activity could be occurring in the BSTPM, and projections to the AH would then initiate aggressive behavior. Another possibility for the initiation of agonistic behavior in defeated hamsters is that CRFR1 binding is occurring in the BSTA, which may have projections to the appropriate subregions of the BSTP to block aggressive behavior in previously defeated hamsters while indirectly activating defensive behavior via the BSTPL (or the BSTPL may be directly activated by concomitant binding at CRFR1 in that subregion).

Corticotropin Releasing Factor Receptors and Agonistic Behavior in Syrian Hamsters

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by

Alicia N. Faruzzi

Committee:

Dr. Kim L. Huhman, Chair

Dr. H. Elliott Albers, Member

Dr. Aras Petrulis, Member

Dr. Larry J. Young, Member

Date

Dr. Mary Morris
Department Chair